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The involvement of the novel proteins, perilipin, ATGL, and CGI-58, in lipolysis of early  
and mid lactation Holstein cows

by

Dawn A. Elkins

A thesis submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of  
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Program of Study Committee:  
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Ames, Iowa

2008

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**ABSTRACT**

Perilipin, adipose triglyceride lipase (ATGL), and comparative gene identity-58 (CGI-58) are novel proteins that greatly effect lipid catabolism in rodents and humans. During early lactation, dairy cows often enter negative energy balance when milk production requirements exceed energy intake, and must mobilize adipose tissue to meet these needs. Severe and prolonged negative energy balance can lead to reduced fitness. Perilipin, ATGL and CGI-58 protein abundance was quantified to determine the relationship of these proteins with adipose tissue mobilization of early (5-14 days in milk [DIM]; n=11) compared to mid (176-206 DIM; n=9) lactation cows. Phosphorylation of perilipin was increased in early lactation and significantly correlated with lipolytic indicators in early and mid lactation. ATGL was increased in mid lactation and CGI-58 was similar across stages of lactation. These results suggest phosphorylation of perilipin occurs during stimulated and basal lipolysis; whereas ATGL may function as a basal lipase.

## **CHAPTER 1. GENERAL INTRODUCTION**

### **1.1 INTRODUCTIONS**

#### **1.1.1 Role of Energy Balance in the Dairy Industry**

Milk production of cows born in 2006 compared to those born in 1957 increased by 6,174 kg (AIPL, 2008a). Significant increases in milk production have been accompanied by increases in energy demands. When these energy demands are not met, the cows enter negative energy balance. It has been reported that negative energy balance during early lactation can be as severe as -43 MJ (Bradford and Allen, 2008) or as mild as -19 MJ (Kessel et al., 2008). When negative energy balance is severe or prolonged, cows are at greater risk for decreased fitness traits.

Reduced fitness has been measured by various traits. The effects of negative energy balance on reproductive fitness have been a subject of much research. Methods of estimating energy balance have ranged from milk production and body condition scores to measurement of feed intake and milk production to calculate net energy balance. Milk production records are readily available on many farms and have been used to estimate energy expenditure. Due to the availability of milk production records, they have been used by many studies to determine the effects of energy expenditure on reproductive fitness. Milk yield has been negatively correlated with the number of days until first ovulation as measured by luteal activity (Veerkamp et al., 2000). Interestingly, increases in milk production have also been associated with multiple ovulations as a possible result of varying hormone levels in

high- versus average- producing cows (Lopez et al., 2005). Body condition scores have been used as an on the farm indicator of energy balance, but these are subjective scores that are delayed from *in vivo* conditions (Oikonomou et al., 2008). Body condition scores have been associated with decreased ovulation (Lopez et al., 2005) and fertility (De Haas et al., 2007). Finally, direct measurements of energy balance have been associated with increases in the number of days open (Patton et al., 2007) and decreased pregnancy rates (Reist et al., 2003).

Negative energy balance and metabolic diseases have been studied for many years; however, advances in technology have now associated negative energy balance with compromised immunity. As mentioned, initial studies linked negative energy balance with metabolic disease due to over conditioning of cows prior to parturition, resulting in an increased risk for ketosis, fatty liver disease, and milk fever. Immune function can become compromised with negative energy balance, as seen by decreases of natural antibody production by cows experiencing negative energy balance (Hammon et al., 2006; van Knegsel et al., 2007). However, direct inoculation of bacteria (*E. coli*) or farm records have failed to link milk production with compromised immune function (Collard et al., 2000; Kornalijnslijper et al., 2003). Therefore, even though milk production has been used as a measure of energy expenditure, only energy balance is associated decreased immune function.

Negative energy balance affects many different systems which lack sufficient energy requirements to function properly. This effect can not be only short term, in the case of reproduction and immune function, but can affect longevity. In 2008, Animal Improvement Production Laboratory reported a decrease of 7.17 months in

the production life of a dairy cow born between 1960-2005 (AIPL, 2008b). Many factors may contribute to decreased longevity, including an increase in somatic cell counts during later lactations that are associated with low body condition scores during first lactation (Banos et al., 2006), decreased growth rates as a result of increased milk production demands on first lactation cows (Wall et al., 2007), or increased laminitis as a result of decreased energy balance (Collard et al., 2000).

Clearly, negative correlations exist between energy balance and fitness traits. Decreases in immune function due to negative energy balance can increase risks of mastitis, milk fever and ketosis, which cost the United States dairy industry close to \$2.6 billion (Sonstegard, 2003). In addition, involuntary culling of cows due to reductions in other fitness traits such as lameness, reproductive inefficiency, and high somatic cell counts cost the dairy industry considerably. The reason for the decrease in fitness is likely due to multiple factors, but clearly negative energy balance in cows is one cause. One critical factor that is directly influenced by energy balance is the regulation of energy mobilization by adipose tissue. Therefore, determining the mechanisms of adipose tissue mobilization is essential to understanding the biology of negative energy balance.

### **1.1.2 Mobilization of Adipose Tissue**

Adipose tissue was traditionally viewed as a reservoir of densely stored excess energy available for use in times of energy shortage. Over the past decade, research has reconstructed this idea of the function of adipose tissue. Original roles of adipose tissue pertained primarily to energy storage or heat regulation. However, adipose tissue is now recognized as a dynamic tissue capable of autocrine,

endocrine and immunological functions. This realization has sparked renewed interest in adipose tissue and its regulation. As knowledge of adipose tissue has evolved, so have the mechanisms of lipid mobilization.

A major pathway involved in the catabolism of triacylglycerides during negative energy balance is the protein kinase A (**PKA**) pathway. As traditionally viewed, stimulation of lipolysis is initiated through catecholamines (epinephrine, norepinephrine, etc.) binding to beta adrenergic receptors ( **$\beta$ -AR**) which activate adenylylate cyclase via G-proteins (See Figure 1), resulting in the conversion of adenosine triphosphate (**ATP**) to cyclic adenosine monophosphate (Campagna et al.). The increase in cAMP results in the activation PKA which phosphorylates cytosolic hormone sensitive lipase (**HSL**) allowing for HSL translocation to the lipid droplet. Triacylglycerides are catabolized by phosphorylated HSL at the lipid droplet surface resulting in the release free fatty acids (**FFA**) and glycerol to the circulation.

More recently, proteins associated with the lipid droplet have gained recognition as important modulators of lipolysis. In the basal state, perilipin coats the lipid droplet preventing the interaction HSL with the lipid droplet. When phosphorylated, perilipin can facilitate catabolism of triacylglycerides by allowing phosphorylated HSL to interact with the lipid droplet (Greenberg et al., 1991). A novel lipase was discovered when HSL ablated mice remained lean, indicating the presence of another active lipase in rodents (Zimmermann et al., 2004). This lipase was identified as adipose triglyceride lipase (ATGL) which is both cytosolic and lipid droplet bound (Granneman et al., 2007). Adipose triglyceride lipase hydrolyzes triacylglycerides producing diacylglycerides (Zimmermann et al., 2004).

Comparative gene identity-58 (CGI-58) is another lipid droplet associated protein whose role in lipolysis is not fully understood. CGI-58 translocates away from the lipid droplet upon stimulus (Yamaguchi et al., 2007) and associates with ATGL (Granneman et al., 2007). Many questions still remain about the interaction of these novel proteins with PKA-dependent lipolysis. To date, perilipin mRNA abundance has been characterized in dairy cattle (Sumner and McNamara, 2007), but there are no published reports describing the protein abundance or phosphorylation of perilipin, or the presence of ATGL or CGI-58 in dairy cattle.

## **1.2 OBJECTIVES**

The overall goal of this study was to investigate the novel proteins, perilipin, ATGL, and CGI-58 in relation to lipolysis in lactating dairy cattle. The first objective was to explore the relationship of perilipin at different stages of lactation, by determining the level at which perilipin (mRNA, protein, or phosphorylation) abundance was associated with stage of lactation. Additionally, perilipin abundance (mRNA, protein and phosphorylation) was associated with serum lipolytic indicators in early (5-14 DIM) and mid (176-206 DIM) lactation cows. We hypothesized that phosphorylation of perilipin would be increased during early (5-14 DIM) compared to mid (176-206 DIM) lactation, and correlated with lipolytic indicators.

The second objective was to determine the relationship of the novel lipase, ATGL, and its activator, CGI-58, with stages of lactation by determining if ATGL and/or CGI-58 protein abundance differed between early (5-14 DIM) and mid (176-206 DIM) lactation, and the relationship of ATGL and CGI-58 with serum lipolytic indicators within early and mid lactation cows. We hypothesized that both ATGL

and CGI-58 abundance would be increased during early compared to mid lactation, and correlated with lipolytic indicators.

The third objective was to further characterize differences between early and mid lactation cows by determining if isolated adipocytes from early (5-14 DIM) and mid (176-206 DIM) lactation cows responded differently to a non-selective  $\beta$ -agonist. We also wanted to determine if adipocyte cell size differs between early and mid lactation. We hypothesized that early lactation cows would be more sensitive to  $\beta$ -agonist and have decreased average adipocyte size compared to mid lactation cows.

## 1.3 REVIEW OF RELEVANT LITERATURE

### 1.3.1 Beta-Adrenergic Receptors

Catecholamines initiate the PKA pathway in adipocytes by binding beta adrenergic receptors ( **$\beta$ -AR**). Three isoforms of  $\beta$ -AR ( $\beta$ -AR<sub>1</sub>,  $\beta$ -AR<sub>2</sub>,  $\beta$ -AR<sub>3</sub>) are present in cattle. Activity of these isoforms varies among species.

Of the  $\beta$ -AR,  $\beta$ -AR<sub>2</sub> is expressed most abundantly in adipose tissue of cattle (Sumner and McNamara, 2007). In dairy cattle,  $\beta$ -AR<sub>2</sub> mRNA expression was highest at 90 DIM corresponding with an increase in NEFA (Sumner and McNamara, 2007), consistent with a role of  $\beta$ -AR<sub>2</sub> in lipolysis. In contrast,  $\beta$ -AR<sub>1</sub> and  $\beta$ -AR<sub>3</sub> are abundantly expressed in brown adipose tissue, which is present in calves and supports thermoregulation (Bareille and Faverdin, 1996; Ferlay and Chilliard, 1999); Casteilla et al., 1994). However, mRNA abundance of  $\beta$ -AR<sub>1</sub> and

$\beta$ -AR<sub>3</sub> in white adipose tissue was similar throughout lactation (Sumner and McNamara, 2007).

Phenethanolamines or  $\beta$ -agonists have been used to repartition energy in livestock animals and redirect nutrients away from adipose tissue to muscle (Ricks, 1984). The use of  $\beta$ -agonists result in increased rates of weight gain, improved feed utilization efficiency, increased leanness, and increased dressing percentage (Moody, 2000). To date, two phenethanolamine repartitioning agents have been approved for livestock use. However,  $\beta$ -adrenergic receptors are down-regulated with extended use of  $\beta$ -agonist, and are most effective at partitioning energy away from adipose tissue to muscle for limited times.

### **1.3.2 Hormone Sensitive Lipase**

Hormone sensitive lipase has been well characterized in different models and relative to multiple signal transduction pathways. Most notably, HSL is regulated by the PKA and protein kinase C (**PKC**) pathways. The PKA pathway is activated via  $\beta$ -AR in response to hormonal stimulation, as previously described, whereas the PKC pathway is stimulated by calcium, diacylglycerol, and phospholipids, and is inhibitory to the PKA pathway. Most mechanistic studies of HSL utilized an immortal preadipocyte cell line (3T3-L1), and genetically modified rodent models to characterize phosphorylation sites at sub-cellular locations.

Hormone sensitive lipase resides in the cytosol and is regulated by multiple phosphorylation sites. Phosphorylation of HSL results in the translocation of phosphorylated HSL to the lipid droplet. Serines 563, 659, and 660 are phosphorylated by PKA in response to  $\beta$ -AR stimulation. Although serine 563 was



the first PKA phosphorylation site to be described (Egan et al., 1992), serines 659 and 660 express greater activity (Anthonsen et al., 1998). Other phosphorylation sites, such as serine 600 and 565, undergo phosphorylation via PKA independent mechanisms. For example, serine 565 is phosphorylated by adenosine monophosphate kinase (AMPK), and is thought to be a regulator of basal lipolysis (or lipolysis during neutral energy balance) and possible inhibitor of PKA phosphorylation (Carmen and Victor, 2006; Daval et al., 2005). Phosphorylation of serine sites by PKA induces the translocation (Egan et al., 1992; Miyoshi et al., 2007) of HSL to the lipid droplet within 5 minutes of stimulation (Brasaemle et al., 2000a). At the lipid droplet surface, phosphorylated HSL interacts with phosphorylated perilipin to gain access to lipid substrates (Carmen and Victor, 2006; Fricke et al., 2004; Miyoshi et al., 2006; Moore et al., 2005).

The relationship of HSL to *in vivo* lipolysis has not been well characterized. In humans, polymorphisms of HSL have been associated with increased body mass index (Garenc et al., 2002). Feed restricted sheep and cows exhibited increased mRNA abundance of HSL (Bonnet et al., 1998), and cattle have highest HSL mRNA expression at the time of peak NEFA release (Sumner and McNamara, 2007). Literature from ruminants suggests increased HSL mRNA production during times of energy deficit; however, the phosphorylation of HSL by PKA during times of energy deficit has yet to be characterized.

### **1.3.3 Perilipins**

Perilipins are phosphoproteins associated with the lipid droplet with two isoforms in rodents; a full length form, A, and the shortened form, B. Initially, it was

speculated that the absence or presence of perilipin at the lipid droplet allowed phosphorylated HSL to interact with the lipid droplet (Daval et al., 2005). The presence of perilipin at the lipid droplet was thought to act as a barrier, allowing for increased lipid storage and decreasing the rate of lipolysis (Brasaemle et al., 2000b). Originally, phosphorylation of perilipin by PKA was thought to activate a translocation event where perilipin translocated away from the lipid droplet allowing for catabolism of triacylglycerides by phosphorylated HSL. However, this theory was revised as a result of studies using fluorescence resonance energy transfer (**FRET**) and fluorescent antibodies that discerned the sub-cellular location of perilipin during stimulated and basal lipolysis. Results revealed that perilipin does not translocate away from the lipid droplet upon stimulation of lipolysis, but can enhance lipolytic response by interacting with phosphorylated HSL (Granneman et al., 2007; Moore et al., 2005). These studies have molded the current theory of perilipin as dual purpose protein that can inhibit and enhance lipolysis.

Research exploring the movements of HSL and active phosphorylation sites of perilipin utilized immortalized cells lines. Adipocytes lacking perilipin have a reduction of phosphorylated HSL translocation and a significant reduction in glycerol release (Miyoshi et al., 2006; Sztalryd et al., 2003). Six serine phosphorylation sites reside in perilipin and all have been shown to be phosphorylated by PKA. Mutation of any of these PKA phosphorylation sites reduces lipolytic activity, but still allows phosphorylated HSL to localize to lipid droplet (Miyoshi et al., 2007; Tansey et al., 2003). However, if the C-terminus end serines (4,5,6) are mutated, lipolysis is dramatically reduced compared to other serine phosphorylation sites (Tansey et al.,

2003). The C-terminus end is also essential for ATGL lipolytic response (Miyoshi et al., 2007). The unique relationship between HSL and perilipin reveals a need for the presence of perilipin to aide in the translocation of HSL, but most importantly, that phosphorylation of perilipin might be a pivotal step in regulating lipolysis.

Knock-out mouse models have been used to describe the role of perilipin in lipid accumulation and lipolysis. Perilipin ablated mice had significantly less fat pad mass than wild type mice (Tansey et al., 2001); suggesting perilipin is essential for lipid accumulation. Knock-out perilipin mice had increased NEFA,  $\beta$ -hydroxybutyrate and glycerol release during neutral lipolysis, but decreased NEFA,  $\beta$ -hydroxybutyrate and glycerol release during stimulated lipolysis (Martinez-Botas et al., 2000; Tansey et al., 2001) confirming the dual roles of perilipin as inhibitor and enhancer of lipolytic activity.

Little is known about the degradation of perilipin. Two contrasting studies have been published about perilipin protein breakdown. Xu et al. (2006) used Chinese Hamster Ovarian (**CHO**) cells and observed degradation of perilipin through the ubiquitin-protease pathway. However, Kovsan et al. (2007) used 3T3-L1 pre-adipocytes and observed degradation of perilipin through the lysosomal pathway. CHO cells are not naturally lipid droplet laden and must be induced to store lipids whereas 3T3-L1 cells are pluripotent and upon differentiation can become adipocytes. Therefore, study of cells most indicative of *in vivo* adipocytes suggests the lysosomal pathway to be the most likely pathway of degradation for perilipin.

Perilipin has been linked with lipid accumulation and basal rates of lipolysis in humans. Protein and/or mRNA abundance of perilipin are negatively associated

with body mass (Kern et al., 2004; Wang et al., 2003) and a single nucleotide polymorphism (**SNP**) has been associated with rate of basal lipolysis (Mottagui-Tabar et al., 2003). From these findings and others, researchers have suggested that perilipin does not form a continuous ring around the lipid droplet, but is spaced intermittently. Obesity research suggests that as lipid droplets increase in size, the gaps between perilipin proteins are expected to increase as a result in either similar or decreased protein abundance, resulting in increased lipolysis.

In dairy cattle, one study has characterized perilipin mRNA expression through the transition period. Sumner and McNamara observed increases in perilipin mRNA with the onset of lactation, and expression peaked at 90 DIM along with the peak in serum NEFA (2007). These results suggest perilipin mRNA expression in cattle increases with adipose tissue mobilization. However, in the absence of perilipin protein abundance data, many questions remain about perilipin mRNA, protein, and phosphorylation in differing energy states of dairy cows, and how this effects lipolysis during negative energy balance.

#### **1.3.4 Adipose Triglyceride Lipase**

In 2004, three independent labs studying HSL-ablated mice observed that mice lacking this rate-limiting enzyme of lipolysis were non-obese and had accumulations of diacylglycerides (Jenkins et al., 2004; Villena et al., 2004; Zimmermann et al., 2004). These important observations suggested the presence of an additional lipase, adipose triglyceride lipase (**ATGL**; also referred to as desnutrin, calcium-independent phospholipase A2, and patatin-like phospholipase domain containing 2). Recent studies strongly indicate that ATGL truncations are

involved in Neutral Lipid Storage Disease (**NLSD**), a disease associated with slight to severe myopathy, (Akiyama et al., 2007; Campagna et al., 2008).

Through cell culture and clinical cases of NLSD without ichthyosis, the C-terminus end of ATGL has been characterized. Initial studies found the C-terminus end of ATGL is the active terminus (Schweiger et al., 2008) and that it interacts with the N-terminus of CGI-58 (Granneman et al., 2007). The level of activity of this C-terminus region of ATGL varies with species (Schweiger et al., 2008). For example, the C-terminus end of ATGL in mice is less active compared to the C-terminus end of human ATGL (Schweiger et al., 2008). In human research, mutations of the C-terminus end have been associated with the degree of severity of NLSD. Five mutations and early terminations of ATGL found in NLSD patients, surprisingly, increased activity relative to wild-type ATGL. Additionally, ATGL lacking the full C-terminus end had increased *in vitro* triglyceride hydrolase activity, indicating increased lipolytic activity potential (Schweiger et al., 2008). ATGL localizes to both the cytosol and the lipid droplet. However, ATGL lacking the full C-terminus end localized primarily to the cytosol, compared to wild-type ATGL will that localized primarily to the lipid droplet in COS-7 cells (Schweiger et al., 2008). Interaction with CGI-58 is needed to move ATGL to the lipid droplet where it can interact with lipid substrates.

Knock-out ATGL mice were the first models used to characterize functions of ATGL. Haemmerle et al. reported rodent models lacking ATGL have increased adipose tissue mass, including increases in adipose tissue in skeletal muscle and cardiac muscle (2006). These results suggest a role for ATGL in the regulation of

lipid accumulation in multiple tissues. The increased adipose tissue accumulation in cardiac muscle decreases survival of knock-out ATGL mice (Haemmerle et al., 2006). Other characteristics of ATGL ablated mice include a decreased response to stimulation by known  $\beta$ -agonists, and an inability to maintain body temperature under cold (4°C) exposure (Haemmerle et al., 2006). Decreased response to  $\beta$ -agonist stimulation and accumulation of diacylglycerides in HSL ablated mice suggest a role for ATGL in stimulated lipolysis. However, the inability of ATGL ablated mice to maintain body temperature could be due to significant transcriptional changes in brown adipose tissue, suggesting changes in uncoupling proteins (Pinet et al., 2008).

Research involving human adipocytes has provided new insight into the role of ATGL in lipolysis. A study exploring the role of ATGL in human pre-adipocytes used small interfering RNA (**siRNA**) to knock down HSL or ATGL protein. Results of this study differed from those found in rodent knock out models. Use of siRNA against HSL and ATGL in human adipocyte cells resulted in decreased basal lipolysis, but only siRNA of HSL decreased stimulated triglyceride hydrolase activity (Ryden et al., 2007). This indicates that both lipases are utilized in basal lipolysis, but that only HSL is needed for stimulated lipolysis in human adipocytes.

Cell culture of 3T3-L1 preadipocytes demonstrates similar patterns of HSL and ATGL expression in responses to various stimuli. Decreased HSL and ATGL expression was observed with isoproterenol, tumor necrosis factor  $\alpha$ , and insulin stimulation in 3T3-L1 (Kralisch et al., 2005) suggesting HSL and ATGL are similarly regulated. However, ATGL expression is partially rescued from insulin down

regulation with p44/42 MAP kinase (Kralisch et al., 2005) suggesting additional regulation by other mechanisms.

Adipose triglyceride lipase is correlated with HSL (Steinberg et al., 2007), but is not present in pre-adipocytes (Kershaw et al., 2007). Peroxisome proliferator-activated receptor  $\gamma$  (**PPAR $\gamma$** ) is a transcription factor that regulates differentiation of preadipocytes to adipocytes and increases as pre-adipocytes mature into adipocytes. As levels of PPAR  $\gamma$  increase in pre-adipocytes, ATGL also increases (Kershaw et al., 2007). Therefore, as adipocytes mature, ATGL increases with the ability to work in tandem with HSL to hydrolyze triacylglycerides.

ATGL abundance is different in humans compared to mice and is not affected by body mass index in humans (Mairal et al., 2006; Ryden et al., 2007; Steinberg et al., 2007). However, differences in ATGL expression according to adipose depot have been observed. Visceral adipose ATGL expression is not affected by body mass index, whereas, subcutaneous adipose tissue ATGL expression is associated with body mass index (Steinberg et al., 2007). Adipose triglyceride lipase expression is affected by age instead of feed status in rats (Caimari et al., 2008) which could be due to increases in PPAR $\gamma$  expression. However, porcine ATGL expression was increased with feed restriction, but porcine ATGL expression was still decreased by  $\beta$ -agonist (Deiullis et al., 2008). At this time, there are no published data describing ATGL in dairy cattle.

### **1.3.5 Comparative Gene Identity-58**

Comparative Gene Identity-58 (**CGI-58**) was named according to its method of identification. Lai et al. compared *Caenorhabditis elegans* and human transcripts

and found 150 of these novel conserved transcripts (2000). CGI-58 is an esterase/lipase/thioesterase subfamily member of the  $\alpha/\beta$ -hydrolase fold enzyme family. From physical attributes, CGI-58 was also named  $\alpha/\beta$ -hydrolase domain 5 (**ABHD5**). Herein, it will be notated as CGI-58 or Comparative Gene Identity-58. Following the identification of CGI-58, its association was quickly established that NLSD with ichthyosis, also known as Chanarin-Dorfman syndrome. Eight mutations of CGI-58 at the C-terminus end result in causative mutations in NLSD with ichthyosis due to truncation or early termination. Existing research involving CGI-58 focuses primarily on its interactions with perilipin on the lipid droplet, and with ATGL in the cytosol (Granneman et al., 2007).

Comparative Gene Identity-58 interacts with perilipin and adipocyte differentiation-related protein (**ADRP**) at the lipid droplet. Using Glutathione-S-transferase (**GST**) pull-down techniques, Yamaguchi et al. were able to demonstrate binding of CGI-58 with both perilipin and ADRP (2006). Yamaguchi et al. (2006) and Subramanian et al. (2004) utilized immunofluorescence to evaluate the localization of CGI-58 and perilipin. Immunofluorescence of CGI-58 in 3T3-L1 pre-adipocytes reveals that CGI-58 is cytosolic prior to full differentiation (Yamaguchi et al., 2006). In differentiated 3T3-L1 cells, CGI-58 and perilipin had similar localization patterns in basal states at the lipid droplet. CGI-58 will localized at the lipid droplet with perilipin lacking PKA sites 4, 5, and 6, and full to partial amounts of CGI-58 will translocate to the cytosol (Yamaguchi et al., 2006). PKA sites 4, 5 and 6 are essential for the phosphorylation of perilipin and subsequent docking with phosphorylated HSL leading to lipolysis. However, phosphorylation sites of perilipin are not essential for



CGI-58 to function, but may prevent full activity by allowing partial translocation of CGI-58 from the lipid droplet. However, a mutation of perilipin that lacks amino acids 382-405 (closely related to perilipin B) fail to initially localize CGI-58 to the lipid droplet (Yamaguchi et al., 2006). These findings indicate that phosphorylation of perilipin by PKA is not needed for translocation of CGI-58 to the cytosol, but the amino acids 382-405 are essential for CGI-58 activity. However, phosphorylation by PKA instigates translocation of CGI-58 to the cytosol.

CGI-58 translocates from the lipid droplet to the cytosol where it interacts with cytosolic ATGL to activate ATGL, as demonstrated by immunofluorescence and FRET in 3T3-L1 adipocytes (Granneman et al., 2007). The addition of CGI-58 to COS-7 lipid-laden cells expressing only ATGL resulted in increased free fatty acid release (Lass et al., 2006). In ATGL ablated mice, the addition of CGI-58 decreased lipolytic activity of HSL (Schweiger et al., 2006). This indicates that only ATGL activity is increased with the addition of CGI-58.

Few characteristics of CGI-58 are known outside of CGI-58 interaction with perilipin and ATGL. CGI-58 contains coding regions associated with PKA phosphorylation. However, exploration of PKA phosphorylation sites in CGI-58 revealed that increased cAMP and PKA activation failed to stimulate translocation of CGI-58 (Subramanian et al., 2004; Yamaguchi et al., 2007). Also, CGI-58, unlike ATGL, is expressed in the cytosol in pre-adipocytes, and knock-down CGI-58 cells exhibit decreased basal and stimulated lipolysis (Yamaguchi et al., 2007).

### 1.3.6 Characteristics of Bovine Adipose Tissue

Lipolytic measurements of adipose tissue have been used to characterize lipolysis in dairy cattle. Glycerol release from adipose tissue explant cultures stimulated by epinephrine have shown that  $\beta$ -adrenergic receptors are more sensitive in tissue undergoing lipolytic activity (Sumner and McNamara, 2007). However, large variation is associated with tissue explant where even patterns of sensitivity deviate from the expected. For example, adipose tissue explant cultures stimulated by isoproterenol exhibited increased  $\beta$ -adrenergic sensitivity during the dry period compared to early lactation (McNamara and Hillers, 1986).

When adipocytes undergo lipolysis, they are known to decrease in size as the lipid droplets are dispersed. In transition cows, adipocytes are known to decrease in average adipocyte size as lipolytic activity increases (McNamara et al., 1992). At this time, exploration of adipocyte size in dairy cows in mid to late lactation is unknown, but would be expected to be increased due to excess energy during this time.

## 1.4 REFERENCES

- AIPL. 2008a. Trend in milk bv for holstein or red and white No. 2008. Animal Improvement Programs Laboratory.
- AIPL. 2008b. Trend in productive life for holstein or red and white No. 2008. Animal Improvement Program Laboratory.
- Akiyama, M. et al. 2007. Novel duplication mutation in the patatin domain of adipose triglyceride lipase (pnpla2) in neutral lipid storage disease with severe myopathy. Muscle Nerve 36: 856-859.
- Anthonsen, M. W., L. Ronnstrand, C. Wernstedt, E. Degerman, and C. Holm. 1998. Identification of novel phosphorylation sites in hormone-sensitive lipase that

- are phosphorylated in response to isoproterenol and govern activation properties in vitro. *J Biol Chem* 273: 215-221.
- Banos, G., M. P. Coffey, E. Wall, and S. Brotherstone. 2006. Genetic relationship between first-lactation body energy and later-life udder health in dairy cattle. *J Dairy Sci* 89: 2222-2232.
- Bareille, N., and P. Faverdin. 1996. Lipid metabolism and intake behavior of dairy cows: Effects of intravenous lipid and beta-adrenergic supplementation. *J Dairy Sci* 79: 1209-1220.
- Bonnet, M. et al. 1998. Messenger rnas encoding lipoprotein lipase, fatty acid synthase and hormone-sensitive lipase in the adipose tissue of underfed-refed ewes and cows. *Reprod Nutr Dev* 38: 297-307.
- Bradford, B. J., and M. S. Allen. 2008. Negative energy balance increases periprandial ghrelin and growth hormone concentrations in lactating dairy cows. *Domest Anim Endocrinol* 34: 196-203.
- Brasaemle, D. L., D. M. Levin, D. C. Adler-Wailes, and C. Londos. 2000a. The lipolytic stimulation of 3t3-l1 adipocytes promotes the translocation of hormone-sensitive lipase to the surfaces of lipid storage droplets. *Biochim Biophys Acta* 1483: 251-262.
- Brasaemle, D. L. et al. 2000b. Perilipin a increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. *J Biol Chem* 275: 38486-38493.
- Caimari, A., P. Oliver, and A. Palou. 2008. Impairment of nutritional regulation of adipose triglyceride lipase expression with age. *Int J Obes (Lond)*.
- Campagna, F. et al. 2008. Novel mutations in the adipose triglyceride lipase gene causing neutral lipid storage disease with myopathy. *Biochem Biophys Res Commun*.
- Carmen, G. Y., and S. M. Victor. 2006. Signalling mechanisms regulating lipolysis. *Cell Signal* 18: 401-408.

- Collard, B. L., P. J. Boettcher, J. C. Dekkers, D. Petitclerc, and L. R. Schaeffer. 2000. Relationships between energy balance and health traits of dairy cattle in early lactation. *J Dairy Sci* 83: 2683-2690.
- Daval, M. et al. 2005. Anti-lipolytic action of amp-activated protein kinase in rodent adipocytes. *J Biol Chem* 280: 25250-25257.
- De Haas, Y., L. L. Janss, and H. N. Kadarmideen. 2007. Genetic correlations between body condition scores and fertility in dairy cattle using bivariate random regression models. *J Anim Breed Genet* 124: 277-285.
- Deiuliis, J. A. et al. 2008. Developmental, hormonal, and nutritional regulation of porcine adipose triglyceride lipase (atgl). *Lipids* 43: 215-225.
- Egan, J. J. et al. 1992. Mechanism of hormone-stimulated lipolysis in adipocytes: Translocation of hormone-sensitive lipase to the lipid storage droplet. *Proc Natl Acad Sci U S A* 89: 8537-8541.
- Ferlay, A., and Y. Chilliard. 1999. Effects of the infusion of non-selective beta-, and selective beta1- or beta2-adrenergic agonists, on body fat mobilisation in underfed or overfed non-pregnant heifers. *Reprod Nutr Dev* 39: 409-421.
- Fricke, K., A. Heitland, and E. Maronde. 2004. Cooperative activation of lipolysis by protein kinase a and protein kinase c pathways in 3t3-l1 adipocytes. *Endocrinology* 145: 4940-4947.
- Garenc, C. et al. 2002. The hormone-sensitive lipase gene and body composition: The heritage family study. *Int J Obes Relat Metab Disord* 26: 220-227.
- Granneman, J. G. et al. 2007. Analysis of lipolytic protein trafficking and interactions in adipocytes. *J Biol Chem* 282: 5726-5735.
- Greenberg, A. S. et al. 1991. Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. *J Biol Chem* 266: 11341-11346.
- Haemmerle, G. et al. 2006. Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. *Science* 312: 734-737.

- Hammon, D. S., I. M. Evjen, T. R. Dhiman, J. P. Goff, and J. L. Walters. 2006. Neutrophil function and energy status in holstein cows with uterine health disorders. *Vet Immunol Immunopathol* 113: 21-29.
- Jenkins, C. M. et al. 2004. Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase a2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. *J Biol Chem* 279: 48968-48975.
- Kern, P. A., G. Di Gregorio, T. Lu, N. Rassouli, and G. Ranganathan. 2004. Perilipin expression in human adipose tissue is elevated with obesity. *J Clin Endocrinol Metab* 89: 1352-1358.
- Kershaw, E. E. et al. 2007. Ppargamma regulates adipose triglyceride lipase in adipocytes in vitro and in vivo. *Am J Physiol Endocrinol Metab* 293: E1736-1745.
- Kessel, S. et al. 2008. Individual variability in physiological adaptation to metabolic stress during early lactation in dairy cows kept under equal conditions. *J Anim Sci*.
- Kornalijslijper, E. et al. 2003. The effect of milk production level on host resistance of dairy cows, as assessed by the severity of experimental escherichia coli mastitis. *Vet Res* 34: 721-736.
- Kovsan, J., R. Ben-Romano, S. C. Souza, A. S. Greenberg, and A. Rudich. 2007. Regulation of adipocyte lipolysis by degradation of the perilipin protein: Nelfinavir enhances lysosome-mediated perilipin proteolysis. *J Biol Chem* 282: 21704-21711.
- Kralisch, S. et al. 2005. Isoproterenol, tnfa, and insulin downregulate adipose triglyceride lipase in 3t3-l1 adipocytes. *Mol Cell Endocrinol* 240: 43-49.
- Lai, C. H., C. Y. Chou, L. Y. Ch'ang, C. S. Liu, and W. Lin. 2000. Identification of novel human genes evolutionarily conserved in caenorhabditis elegans by comparative proteomics. *Genome Res* 10: 703-713.

- Lass, A. et al. 2006. Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by cgi-58 and defective in chnarin-dorfman syndrome. *Cell Metab* 3: 309-319.
- Lopez, H., D. Z. Caraviello, L. D. Satter, P. M. Fricke, and M. C. Wiltbank. 2005. Relationship between level of milk production and multiple ovulations in lactating dairy cows. *J Dairy Sci* 88: 2783-2793.
- Mairal, A., D. Langin, P. Arner, and J. Hoffstedt. 2006. Human adipose triglyceride lipase (pnpla2) is not regulated by obesity and exhibits low in vitro triglyceride hydrolase activity. *Diabetologia* 49: 1629-1636.
- Martinez-Botas, J. et al. 2000. Absence of perilipin results in leanness and reverses obesity in *lepr(db/db)* mice. *Nat Genet* 26: 474-479.
- McNamara, J. P., B. Becker-Khaleel, and K. L. Parmley. 1992. Quantitative relationships between cyclic adenosine-3',5'-monophosphate and lipolysis in adipose tissue during the peripartum period. *J Dairy Sci* 75: 1901-1913.
- McNamara, J. P., and J. K. Hillers. 1986. Regulation of bovine adipose tissue metabolism during lactation. 2. Lipolysis response to milk production and energy intake. *J Dairy Sci* 69: 3042-3050.
- Miyoshi, H. et al. 2007. Control of adipose triglyceride lipase action by serine 517 of perilipin a globally regulates protein kinase a-stimulated lipolysis in adipocytes. *J Biol Chem* 282: 996-1002.
- Miyoshi, H. et al. 2006. Perilipin promotes hormone-sensitive lipase-mediated adipocyte lipolysis via phosphorylation-dependent and -independent mechanisms. *J Biol Chem* 281: 15837-15844.
- Moody, D. E., D.L Hancock and D.B. Anderson. 2000. Chapter 4. Phenethanolamine repartitioning agents In: J. P. F. D'Mello (ed.) *Farm animal metabolism and nutrition*. p 65-96. CABI Publishing, New York.
- Moore, H. P., R. B. Silver, E. P. Mottillo, D. A. Bernlohr, and J. G. Granneman. 2005. Perilipin targets a novel pool of lipid droplets for lipolytic attack by hormone-sensitive lipase. *J Biol Chem* 280: 43109-43120.

- Mottagui-Tabar, S. et al. 2003. Evidence for an important role of perilipin in the regulation of human adipocyte lipolysis. *Diabetologia* 46: 789-797.
- Oikonomou, G., G. E. Valergakis, G. Arsenos, N. Roubies, and G. Banos. 2008. Genetic profile of body energy and blood metabolic traits across lactation in primiparous holstein cows. *J Dairy Sci* 91: 2814-2822.
- Patton, J. et al. 2007. Relationships among milk production, energy balance, plasma analytes, and reproduction in holstein-friesian cows. *J Dairy Sci* 90: 649-658.
- Pinent, M. et al. 2008. Differential transcriptional modulation of biological processes in adipocyte triglyceride lipase and hormone-sensitive lipase-deficient mice. *Genomics* 92: 26-32.
- Reist, M. et al. 2003. Postpartum reproductive function: Association with energy, metabolic and endocrine status in high yielding dairy cows. *Theriogenology* 59: 1707-1723.
- Ricks, C. A., P.K. Baker and R.H. Dalrymple. 1984. Use of repartitioning agents to improve performance and body composition of meat animals. In: *Reciprocal Meat Conference Proceedings*. p 5-11.
- Ryden, M. et al. 2007. Comparative studies of the role of hormone-sensitive lipase and adipose triglyceride lipase in human fat cell lipolysis. *Am J Physiol Endocrinol Metab* 292: E1847-1855.
- Schweiger, M. et al. 2008. The c-terminal region of human adipose triglyceride lipase affects enzyme activity and lipid droplet binding. *J Biol Chem* 283: 17211-17220.
- Schweiger, M. et al. 2006. Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism. *J Biol Chem* 281: 40236-40241.
- Sonstegard, T., R. Li and C. Van Tassell. 2003. Identification, validation and fine-mapping of quantitative trait loci in dairy cattle No. 2008.
- Steinberg, G. R., B. E. Kemp, and M. J. Watt. 2007. Adipocyte triglyceride lipase expression in human obesity. *Am J Physiol Endocrinol Metab* 293: E958-964.

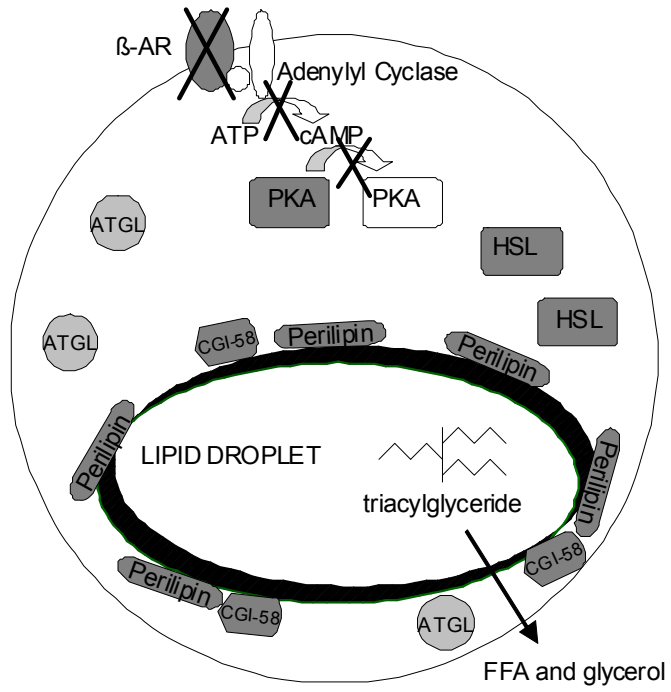
- Subramanian, V. et al. 2004. Perilipin a mediates the reversible binding of cgi-58 to lipid droplets in 3t3-l1 adipocytes. *J Biol Chem* 279: 42062-42071.
- Sumner, J. M., and J. P. McNamara. 2007. Expression of lipolytic genes in the adipose tissue of pregnant and lactating holstein dairy cattle. *J Dairy Sci* 90: 5237-5246.
- Sztalryd, C. et al. 2003. Perilipin a is essential for the translocation of hormone-sensitive lipase during lipolytic activation. *J Cell Biol* 161: 1093-1103.
- Tansey, J. T. et al. 2003. Functional studies on native and mutated forms of perilipins. A role in protein kinase a-mediated lipolysis of triacylglycerols. *J Biol Chem* 278: 8401-8406.
- Tansey, J. T. et al. 2001. Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. *Proc Natl Acad Sci U S A* 98: 6494-6499.
- van Knegsel, A. T. et al. 2007. Natural antibodies related to energy balance in early lactation dairy cows. *J Dairy Sci* 90: 5490-5498.
- Veerkamp, R. F., J. K. Oldenbroek, H. J. Van der Gaast, and J. H. Van der Werf. 2000. Genetic correlation between days until start of luteal activity and milk yield, energy balance, and live weights. *J Dairy Sci* 83: 577-583.
- Villena, J. A., S. Roy, E. Sarkadi-Nagy, K. H. Kim, and H. S. Sul. 2004. Desnutrin, an adipocyte gene encoding a novel patatin domain-containing protein, is induced by fasting and glucocorticoids: Ectopic expression of desnutrin increases triglyceride hydrolysis. *J Biol Chem* 279: 47066-47075.
- Wall, E., M. P. Coffey, and S. Brotherstone. 2007. The relationship between body energy traits and production and fitness traits in first-lactation dairy cows. *J Dairy Sci* 90: 1527-1537.
- Wang, Y. et al. 2003. Perilipin expression in human adipose tissues: Effects of severe obesity, gender, and depot. *Obes Res* 11: 930-936.
- Xu, G., C. Sztalryd, and C. Londos. 2006. Degradation of perilipin is mediated through ubiquitination-proteasome pathway. *Biochim Biophys Acta* 1761: 83-90.



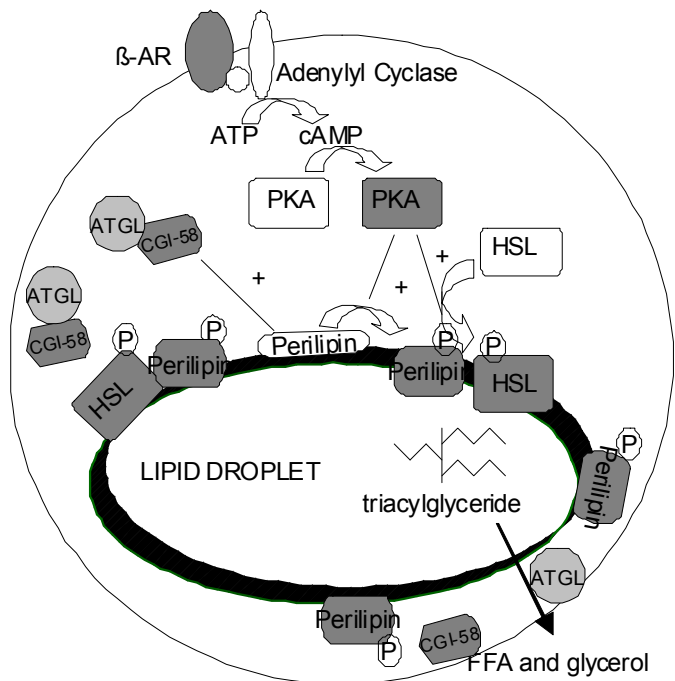
- Yamaguchi, T. et al. 2007. Cgi-58 facilitates lipolysis on lipid droplets but is not involved in the vesiculation of lipid droplets caused by hormonal stimulation. *J Lipid Res* 48: 1078-1089.
- Yamaguchi, T., N. Omatsu, A. Omukae, and T. Osumi. 2006. Analysis of interaction partners for perilipin and adrp on lipid droplets. *Mol Cell Biochem* 284: 167-173.
- Zimmermann, R. et al. 2004. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* 306: 1383-1386.

Figure 1: *Illustration of known mechanism of rodent catabolism. A. Basal state of an adipocyte:* When no stimulation of beta-adrenergic receptor occurs, the PKA pathway is not activated. In this state, HSL remains mostly cytosolic, CGI-58 and perilipin are associated with the lipid droplet associated, and ATGL is both cytosolic and lipid droplet associated. **B. Stimulation of an adipocyte by catecholamines:** When catecholamines bind beta-adrenergic receptors, the PKA pathway is activated leading to the translocation of phosphorylated HSL to the lipid droplet. Phosphorylation of perilipin allows for the interaction of phosphorylated HSL to interact with the triacylglycerides and causes the translocation of CGI-58 away from the lipid droplet to interact with ATGL. *Dark gray filled figures indicate proteins that are activated. Light gray filled figures indicate possible protein activation. Arrows indicated general direction of movement or conversion. Line with + indicate activation and movement in the case of CGI-58 from perilipin. (P) indicates a phosphorylation event.*

A.



B.



## CHAPTER 2. PHOSPHORYLATION OF PERILIPIN IS ASSOCIATED WITH INDICATORS OF LIPOLYSIS IN HOLSTEIN COWS

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### 2.1 ABSTRACT

Perilipin is a protein that coats the lipid droplet of adipocytes. In the basal state, perilipin inhibits lipolysis by restricting access of hormone sensitive lipase (HSL) to the lipid droplet. However, under conditions of stimulated lipolysis, phosphorylated perilipin interacts with HSL to facilitate the interaction of HSL with its lipid substrate. The current experiment was undertaken to determine if changes in perilipin mRNA, protein, or phosphorylation are associated with *in vivo* indicators of lipolysis in lactating dairy cows. Semi-quantitative western blotting and quantitative PCR were used to quantify basal and phosphorylated perilipin in adipose tissue biopsied from cows in early (5 to 14 days in milk [DIM]; n = 11) and mid (176 to 206 DIM; n = 9) lactation. The abundance of perilipin was compared to circulating non-esterified fatty acids and glycerol concentrations assayed from serum as *in vivo* indicators of lipolysis. Expression and phosphorylation of HSL and mRNA abundance of beta-adrenergic receptors were also quantified. As expected, circulating non-esterified fatty acids and glycerol concentrations, beta-adrenergic receptor 2 mRNA abundance, and phosphorylated HSL were significantly greater in

early compared to mid lactation cows. These results demonstrate that cows in early lactation were experiencing increased lipolysis relative to cows in mid lactation. Importantly, it was also found that phosphorylated perilipin, but not total perilipin abundance, was significantly greater in early versus mid lactation cows. Additionally, the abundance of phosphorylated perilipin was correlated to circulating glycerol concentrations during both early and mid lactation. These results suggest that phosphorylated perilipin is a critical factor associated with the regulation of energy mobilization in lactating dairy cows. This has potential impact on the dairy industry because excessive energy mobilization during early lactation is associated with compromised health and fertility traits.

## **2.2 INTRODUCTION**

Dairy cows experience negative energy balance in early lactation when energy intake does not fully offset energy expenditure for lactation and maintenance. As a result, energy substrates are mobilized from adipose and other tissues to correct this energy deficit. Because increases in energy intake have not kept pace with increases in milk production (VanArendonk et al., 1991), modern high-producing cows must meet a significant portion of their energy requirements through mobilization of body energy reserves. Consequently, these energy reserves are unavailable to support fitness traits, and negative energy balance is associated with decreased fertility (Patton et al., 2007; Veerkamp et al., 2000) and increased incidences of metabolic disorders and mastitis (Appuhamy et al, 2007; Banos et al., 2006; Dechow et al., 2004).

Lipolysis is the process that mobilizes energy substrates from adipose tissue through the breakdown of triacylglycerols to fatty acids and glycerol. Stimulation of lipolysis occurs largely through activation of beta adrenergic receptors ( **$\beta$ -AR**). These G-protein coupled receptors stimulate adenylate cyclase to convert ATP to cyclic adenosine monophosphate (**cAMP**). Elevated cAMP activates protein kinase A (**PKA**), leading to the phosphorylation of hormone sensitive lipase (**HSL**). Finally, phosphorylated HSL translocates from the cytosol to the lipid droplet where it hydrolyzes triacylglycerols to nonesterified fatty acids (**NEFA**) and glycerol (Carmen and Victor, 2006; Fricke et al., 2004). In addition to this classic pathway of lipolytic stimulation, perilipin, a lipid droplet associated protein, has been implicated in the regulation of lipolysis via two mechanisms. In the basal state, the presence of perilipin surrounding the lipid droplet inhibits access of HSL to triacylglycerols, thereby inhibiting lipolysis (Tansey et al., 2001; Brasaemle et al., 2000; Martinez-Botas et al., 2000). However, like HSL, perilipin is phosphorylated by PKA during stimulated lipolysis. Phosphorylated perilipin then facilitates interactions between HSL and the lipid droplet, providing increased access of HSL to its lipid substrate (Brasaemle et al., 2004; Tansey et al., 2003, 2001). In this way, phosphorylated perilipin is required for maximal response to lipolytic stimulation.

Despite a growing body of literature describing the role of perilipin in lipolysis, little is known about perilipin in dairy cattle. Thus, the current experiment was undertaken to determine if changes in perilipin are associated with lipolysis in lactating dairy cows. The first objective was to determine if perilipin expression or phosphorylation differs between lactating cows in early versus mid lactation. The

second objective was to determine if variation in either the expression or phosphorylation of perilipin is associated with *in vivo* indicators of lipolysis. In order to accomplish these objectives, perilipin mRNA, protein, and phosphorylation were quantified in cows in early and mid lactation, and compared to circulating NEFA and glycerol concentrations. Additionally, the mRNA abundance of beta-adrenergic receptors 1, 2 and 3 ( $\beta$ -AR<sub>1</sub>,  $\beta$ -AR<sub>2</sub>, and  $\beta$ -AR<sub>3</sub>), and expression and phosphorylation of HSL were quantified to provide comparison with proteins known to be associated with lipolysis.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Animals

Twenty Holstein multiparous cows (lactation 2 through 5) were used in this study, including 11 cows in early lactation, (5 to 14 DIM) and 9 different cows in mid lactation (176 to 206 DIM). None of the 20 cows had experienced mastitis, lameness, or other health disorders during the current lactation. Although energy balance was not measured in this study, others have reported that negative energy balance is common during the initial weeks of lactation (Patton et al., 2007; Banos et al., 2006), and that cows begin returning to positive at approximately 90 DIM (Banos et al., 2006). Thus, the early and mid lactation cows used in this study were expected to differ in energy balance status. Cows in both early and mid lactation were fed the same TMR diet in accordance with their NRC requirements (Clark et al., 2001) and were milked twice daily. Body condition score was determined by a trained evaluator on the day of biopsy, and ranged from 2.75 to 4.5 on a 5 point scale (Wildman et al., 1982). Milk production data were obtained from monthly

DHIA test-day sampling and daily milk records. Milk yield at the time of biopsy was calculated as the average daily milk production from two days before through two days after the day of biopsy. Milk, fat, and protein mature equivalence values generated by DHIA were used to describe production of cows over their complete lactation. These values were estimated after cows were at least 270 DIM, and the DIM did not differ between early and mid lactation cows. The protocol for this experiment was approved by the Iowa State University Institutional Animal Care and Use Committee.

### **2.3.2 Sample Collection**

All samples were collected between May and October, 2007. On sampling days, one early and one mid lactation cow was chosen following their morning milking. Due to animal availability, a biopsy from a single cow was taken on four days. These samples represented three early and one mid lactation cow. Adipose tissue biopsies were taken from the tailhead region. This is a minimally invasive procedure done under local anesthesia, with 2% (v/w) Lidocaine. Following thorough sterilization of the area surrounding the biopsy site, a straight-line incision (approximately 5 cm) was made, and forceps and scalpel were used to remove subcutaneous adipose tissue (approximately 5 g). The incision was closed with surgical staples and covered with antibiotic ointment and vet wrap. The biopsy was immediately frozen in liquid nitrogen and stored at -80°C until further processing. Blood was collected via jugular venipuncture following the biopsy procedure, and serum was filtered and stored at -80°C.



### **2.3.3 Serum Assays**

Serum was assayed for glycerol using Free Glycerol Reagent (Sigma, F6428) and for NEFA using the NEFA-C kit (Wako, 999-75406), both according to manufacturer's protocol. All samples were assayed in triplicate using a 96-well plate format, and data were captured using a Tecan Spectrafluor Plus (Tecan Group Ltd).

### **2.3.4 Semi-quantitative Western Blotting**

Care was taken to dissociate the lipid droplet during protein extraction, as perilipin is associated with the lipid droplet. Protein was extracted from adipose tissue as previously described (Wang et al., 2003), except that protease inhibitor cocktail (Sigma, P8340) and phosphatase inhibitor cocktail set II (Calbiochem, 524627) were used. Protein concentration was determined using BCA Protein Assay kit (Pierce, 23227).

Semi-quantitative western blotting was used to measure perilipin, phosphorylated perilipin, HSL and phosphorylated HSL abundance in adipose tissue biopsies. Samples from all early and mid lactation cows were run together on a single membrane. Sample from an additional cow, chosen arbitrarily, was included in 3 lanes distributed throughout the membrane for use as a relative control. Signal intensities quantified for each experimental sample were expressed relative to the control sample, allowing data to be expressed in relative units. The complete western blotting procedure was repeated on two different membranes to provide duplicate measurements for each sample.

Equal quantities of all samples were mixed with 6X sample loading buffer, boiled for 5 min, and loaded onto 8% SDS gels. Following gel electrophoresis,

samples were transferred to nitrocellulose membranes using a semi-dry transfer unit (Amersham, TE 70). Membranes were blocked in 5% non-fat milk and TBS-T for 2 hours then exposed to primary antibodies at 4°C. After washing, membranes exposed to anti-rabbit IgG Horseradish Peroxidase (**HRP**)-conjugated secondary antibody (GE Healthcare, NA934A). Signal was developed using ECL Plus Western Blotting Detection Reagents (Amersham, RPN 2132), and resulting signal was captured and quantified using an Alpha Imager Gel Documentation System and accompanying software (Alpha Innotech). The quantity of sample loaded, and source and dilution of primary antibody used are given in Table 1.

### **2.3.5 Quantitative Real-time PCR**

Total RNA was extracted from frozen adipose tissue using the RNeasy Lipid Tissue Midi Kit (Qiagen, 75842) with DNase digestion on the column using the RNase-Free DNase Set (Qiagen, 79254). Samples were concentrated using the RNeasy MinElute Cleanup Kit (Qiagen, 74204) and quantified by measuring absorbance at 260 nm on a Nanodrop (Thermo Scientific). All procedures were completed according to manufacturer's protocol.

Complimentary DNA (**cDNA**) was synthesized from 2.5 µg of total RNA using random primers (Invitrogen, 48190-011) and MMLV reverse transcriptase reagents (Invitrogen, 28025-013), according to manufacturer's protocols. In order to generate PCR products at a detectable level, cDNA was synthesized from 1 µg of total RNA using the  $\beta$ -AR<sub>3</sub> reverse PCR primer for gene specific cDNA synthesis.

Quantitative real-time PCR was used to measure mRNA abundance of  $\beta$ -AR<sub>1</sub>,  $\beta$ -AR<sub>2</sub>,  $\beta$ -AR<sub>3</sub>, HSL, and perilipin. The 60S ribosomal protein 32L (**RPL**) mRNA was

quantified for use as a housekeeping gene, as it has been shown to have minimal variation in adipose tissue (D. Spurlock, unpublished data). All samples were run in triplicate on the MyiQ single-color real-time PCR detection system (Bio-Rad) using My iQ SYBR Green Supermix (Bio-Rad, 170-8882) according to the manufacturer's protocol, except that reactions were conducted at half the volume. All PCR primer sequences are provided in Table 2.

The PCR product of each transcript was cloned into the pGem T-easy vector (Promega, A1360) to generate a standard curve for absolute quantification of the target sequences. Plasmid DNA was isolated from transformed *E. coli* cells using a Plasmid Spin Mini kit (Qiagen, 12123), quantified, and diluted to produce standards ranging from  $2 \times 10^2$  to  $2 \times 10^8$  copies per  $\mu\text{l}$ . The cycle threshold determined by quantitative PCR was regressed on the known log of the starting copy number (**LSCN**) of these standards, and the resulting regression line was used to predict the LSCN of all experimental samples. These triplicate LSCN values were used for statistical analyses.

### **2.3.6 Data Analysis**

Differences between early ( $n = 11$ ) and mid ( $n = 9$ ) lactation cows were determined using the PROC MIXED procedure in SAS (SAS Institute, 1999). The mixed model analysis included stage of lactation as a fixed effect, and cow and biopsy day as random effects. Denominator degrees of freedom were determined as described by Kenward and Roger (1997). Protein abundance, NEFA and glycerol data were transformed using the natural log ( $\log_e$ ) to ensure a normal distribution of residuals. Average RPL was used as a covariate for mRNA analyses.

Least squares means and standard errors for stage of lactation were obtained for each trait evaluated. Correlations were calculated within stage of lactation for HSL, perilipin, phosphorylated HSL, phosphorylated perilipin, NEFA, and glycerol using the average of duplicate or triplicate measurements for each cow. Correlations were also calculated between mRNA and protein abundance for HSL and perilipin. Phosphorylated HSL was undetectable in all but one mid lactation cow. Therefore, phosphorylated HSL was compared between early and mid lactation groups using a Chi-square test to determine if the presence or absence of detectable phosphorylated HSL differed between groups.

## 2.4 RESULTS

Cows in early and mid lactation had similar BCS at the time of biopsy ( $3.65 \pm 0.17$  vs.  $3.83 \pm 0.12$  for early and mid lactation cows, respectively,  $P > 0.05$ ), and cows in early lactation were experiencing greater mobilization of adipose tissue energy reserves, as indicated by higher concentrations of circulating NEFA and glycerol ( $P < 0.001$ ; Table 3). Glycerol and NEFA concentrations were highly correlated within early ( $r = 0.80$ ,  $P < 0.01$ ; Table 4), but not mid lactation cows ( $0.39$ ,  $P = 0.30$ ; Table 5). Early and mid lactation cows were producing similar amounts of milk at the time they were biopsied ( $P > 0.05$ ; Table 3). However, cows in the early lactation group produced significantly more milk and milk components over their complete lactation ( $P < 0.001$ , Table 3).

The mRNA abundance of  $\beta$ -AR was evaluated because of their role in stimulating lipolytic activity. Although quantitative PCR is not appropriate for making detailed comparisons of expression across different genes,  $\beta$ -AR<sub>2</sub> appeared to be

expressed at levels that were orders of magnitude greater than  $\beta$ -AR<sub>1</sub> or  $\beta$ -AR<sub>3</sub>. Additionally,  $\beta$ -AR<sub>2</sub> was the only  $\beta$ -AR that was differentially expressed, as mRNA abundance of  $\beta$ -AR<sub>2</sub> in early lactation cows was two-fold greater than that in mid lactation cows ( $P = 0.025$ ; Figure 1, Table 3).

Hormone sensitive lipase was evaluated because it represents the rate-limiting step of lipolysis. Quantitative real-time PCR revealed no difference in HSL mRNA abundance between cows in early versus mid lactation ( $P = 0.192$ ; Figure 2A, Table 3). Likewise, the abundance of HSL protein was similar between these groups ( $P = 0.734$ , Figure 2B, Table 3). However, the correlation between HSL mRNA and protein abundance was not significant ( $r = [-0.15]$ ,  $P = 0.53$ ). In contrast, phosphorylated HSL abundance was greater in early compared to mid lactation cows ( $P = 0.001$ , Figure 2D and E, Table 3).

The relationship between HSL and indicators of lipolysis was further evaluated by determining correlations within the early and mid lactation groups. Within early lactation cows, HSL protein abundance was positively correlated with NEFA and glycerol ( $P < 0.05$ , Table 4). For cows in mid lactation, the correlation between HSL and glycerol remained positive ( $P < 0.05$ ), but no correlation between HSL and NEFA was observed ( $P = 0.02$ ; Table 5). Phosphorylated HSL was positively correlated with glycerol ( $P < 0.05$ ), but not NEFA concentration during early lactation (Table 4). Correlations involving phosphorylated HSL could not be estimated during mid lactation because phosphorylated HSL was undetectable in most of these cows.

The primary objective of this research was to investigate changes in perilipin associated with stage of lactation and lipolytic indicators. No significant differences in perilipin mRNA or protein abundance were found when comparing early to mid lactation cows ( $P > 0.05$ , Table 3, Figure 3A and B). Although this result was similar at the mRNA and protein levels, the correlation between perilipin mRNA and protein was not significant ( $r = [-0.11]$ ,  $P = 0.66$ ). Because of the difference in the role of perilipin in regulating lipolysis depending upon its phosphorylation status, it is essential to evaluate both total and phosphorylated perilipin relative to lipolytic indicators. Although no antibody specific to phosphorylated perilipin was commercially available when this study was done, a phosphor-(Ser/Thr) PKA substrate antibody has been previously demonstrated to clearly identify phosphorylated perilipin in mice (Miyoshi et al., 2006). To verify the suitability of this antibody for detecting bovine phosphorylated perilipin, two western blots were run as previously described to detect phosphorylated perilipin. These blots were then stripped and subsequently exposed to the perilipin antibody. A primary band consistent with the size of perilipin (62 kDa) was detected by the phospho-(Ser/Thr) PKA substrate antibody (Figure 4). This band was subsequently quantified as phosphorylated perilipin, which was found to differ significantly between cows in early versus mid lactation ( $P = 0.0006$ , Figure 3D and E, Table 3).

Correlations of perilipin protein abundance with indicators of lipolysis were non-significant for both early and mid lactation cows ( $P > 0.05$ , Tables 4 and 5). However, phosphorylated perilipin was significantly correlated with NEFA ( $P < 0.05$ )

and glycerol ( $P < 0.05$ ) during early lactation (Table 4), and remained correlated with glycerol during mid lactation ( $P = 0.06$ , Table 5).

## 2.5 DISCUSSION

The data described herein confirm important differences in the abundance of  $\beta$ -AR<sub>2</sub> mRNA and serine phosphorylated HSL with stage of lactation. These results are consistent with existing literature defining a critical role for  $\beta$ -AR in stimulating lipolysis during times of negative energy balance (McNamara, 1988; Jaster and Wegner, 1981), and the view of activated HSL as a critical rate-limiting step of lipolysis. Importantly, the current results define a clear association between phosphorylated perilipin and *in vivo* indicators of lipolysis. This association is apparent based on the significant difference in phosphorylated perilipin abundance between cows in early versus mid lactation, as well as a highly significant correlation between phosphorylated perilipin abundance and circulating glycerol.

The significant correlation between phosphorylated perilipin and glycerol concentration during early lactation is consistent with previous research that utilized knock-out and transgenic mouse models to define a requirement for phosphorylated perilipin during stimulation of lipolysis. Mice lacking perilipin had significantly increased rates of basal lipolysis compared to wild-type mice, as evidenced by less total body fat and increased release of glycerol from isolated adipocytes (Martinez-Botas et al., 2000). These observations support a role for perilipin in limiting access of HSL to the lipid droplet during basal lipolysis. Additionally, the perilipin knock-out mice were unable to increase lipolysis in response to  $\beta$ -AR agonists, suggesting a role for perilipin in mediating a response to lipolytic stimulation. Miyoshi et al., 2006,

further defined the role of perilipin during stimulated lipolysis using mice that expressed mutated perilipin that could not be phosphorylated. In these mice, basal lipolysis was normal, but the mice were unable to respond to lipolytic stimulation via  $\beta$ -AR agonists. Together, these experiments define phosphorylation of perilipin as necessary for increased lipolysis in response to  $\beta$ -AR activation. Because high-producing dairy cows experience negative energy balance during early lactation (Patton et al., 2007; Banos et al., 2006), and negative energy balance stimulates  $\beta$ -AR activation, it is not surprising that increased abundance of phosphorylated perilipin was observed in early compared to mid lactation cows in the current study. Additionally, the correlation between phosphorylated perilipin abundance and glycerol concentration supports a critical role for phosphorylated perilipin in regulating lipolysis.

It is interesting to note that the correlation between phosphorylated perilipin and glycerol concentration was maintained during mid lactation. Cows in mid lactation were sampled because they were expected to be experiencing positive energy balance (Banos et al., 2006) with minimal mobilization of energy reserves from adipose tissue. These expectations were supported by significant reductions in circulating NEFA and glycerol, as well as reduced phosphorylation of the PKA target proteins HSL and perilipin. The correlation between phosphorylated perilipin and glycerol concentration within cows in mid lactation is consistent with a role for phosphorylated perilipin in regulating lipolysis, even in the absence of significant stimulation via classic lipolytic pathways. Such a role was suggested by the observation that inhibition of perilipin dephosphorylation by phosphatase inhibitors



results in increased lipolysis in rat adipocytes (He et al., 2006; Clifford et al., 1998). This increased lipolysis was observed both in the presence and absence of lipolytic stimulation, and was independent of the translocation of HSL to the lipid droplet. He et al. (2006) suggested that phosphorylated perilipin may regulate access of lipases other than HSL to the lipid droplet during basal lipolysis. For example, adipose triglyceride lipase (**ATGL**) has been identified in adipocytes (Zimmermann et al., 2004). Although the significance of ATGL in lipolysis remains unclear, it has been suggested that ATGL is a primary regulator of basal lipolysis in humans (Rydén et al., 2007). Interactions between ATGL and lipid droplet proteins are only partially defined, but may involve phosphorylated perilipin (Granneman et al., 2007). Thus, results from the current study along with existing literature based on cell culture models suggest that phosphorylated perilipin may play an important role in the regulation of lipid metabolism in both the presence and absence of significant lipolytic stimulation.

A correlation between phosphorylated perilipin and circulating NEFA concentration was observed, but only for cows in early lactation. Because early lactation cows were expected to be experiencing negative energy balance, NEFA hydrolyzed through lipolysis were likely being utilized for energy rather than reassembly as triacylglycerols through reesterification pathways. This is supported by a positive correlation between circulating NEFA and glycerol concentrations in early lactating cows. In contrast, cows in mid lactation were likely in a more positive energy balance state and experiencing greater net reesterification. Because circulating NEFA concentrations reflect the balance between lipolysis and

reesterification, and the correlation between NEFA and glycerol was not significant in mid lactation cows, circulating NEFA concentration was likely a poor indicator of lipolysis in the mid lactation cows sampled in this study. Thus, the correlation between phosphorylated perilipin and NEFA in early but not mid lactation cows is consistent with the conclusion that the abundance of phosphorylated perilipin is strongly associated with lipolysis.

Perilipin mRNA abundance has been described in dairy cattle throughout a lactation cycle (Sumner and McNamara, 2007). These authors observed that changes in perilipin mRNA expression followed a similar pattern to changes in circulating NEFA, supporting a relationship between perilipin expression and lipolysis (Sumner and McNamara, 2007). In contrast, perilipin mRNA did not differ between cows in early and mid lactation in the current study, even though NEFA and glycerol differed dramatically. However, important differences between the two experiments exist. First, Sumner and McNamara (2007) sampled adipose tissue during the dry period and at 30, 90 and 270 DIM, whereas the current study evaluated lactating cows at 5-14 and 176-206 DIM. The earlier time point in lactation was chosen for the current study to represent maximal energy mobilization from adipose tissue. Based on existing literature and unpublished data from the herd used in the current study, NEFA and glycerol concentrations spiked at or shortly before calving, and declined rapidly over the first two weeks of lactation (Accorsi et al., 2005). In contrast, cows at 90 DIM are often returning to positive energy balance (Banos et al., 2006). Thus, greater abundance of perilipin mRNA at 90 DIM compared to the dry period (Sumner and McNamara, 2007) may reflect

increasing perilipin as a mechanism contributing to inhibition of lipolysis by limiting access of HSL to the lipid droplet. However, correlations between perilipin mRNA and protein abundance were not significant in the current study, and have been inconsistent in studies in humans (Advedsson et al., 2004; Kern et al., 2004; Wang et al., 2003). Thus, quantification of perilipin protein near the return to positive energy balance is needed to fully understand the results described by Sumner and McNamara (2007). In the current experiment, the mid lactation time point of 176-206 DIM was chosen to represent cows that had returned to positive energy balance, expected to occur at approximately 90 DIM (Banos et al., 2006). Because cows at the Iowa State University Dairy are fed a lower energy diet after approximately 210 DIM, cows were sampled prior to this change to avoid confounding effects of diet. Thus, although the mid lactation time point evaluated in the current study is earlier than the 240 DIM sample utilized by Sumner and McNamara (2007), it is expected that these samples are similar in terms of energy balance and lipid mobilization. Because the previous study compared perilipin mRNA at 240 DIM to samples taken pre-calving, and the current study compared samples between early and mid lactation, it is not clear if results of the experiments are consistent. A second important difference between the two studies is that Sumner and McNamara (2007) utilized repeated samples from the same cows, whereas the current study sampled different cows at early and mid lactation. An advantage of utilizing repeated samples is that potential genetic differences between cows representing different stages of lactation are avoided. However, this strategy has the disadvantage of sampling across time, potentially confounding seasonal

effects with stage of lactation. In the current study, early and mid lactation cows were sampled on the same day so that time of sampling could be accounted for in the statistical analysis. Finally, the selection of cows differed between the previous and current experiments. Sumner and McNamara (2007) utilized both primiparous and multiparous cows selected to be in the top 25% of their herd based on 305d ME. In contrast, the current study only utilized multiparous cows, and did not select cows based on their production potential. By chance, early lactation cows utilized in the current experiment had significantly higher production compared to mid lactation cows.

In summary, this research defines phosphorylated perilipin as a potential mediator of lipolysis. This conclusion is consistent with previous work describing phosphorylated perilipin as necessary for maximal response to lipolytic stimulation (Figure 5). Additionally, the correlation between phosphorylated perilipin and glycerol in mid lactation, when cows are expected to be in positive energy balance, extends this potential role for perilipin. Because PKA phosphorylation is minimal during this time, the regulation of both phosphorylation and dephosphorylation of perilipin may impact the abundance of phosphorylated perilipin and ultimately lipolysis (Figure 5). The current research clearly defines the abundance of phosphorylated perilipin, rather than total perilipin, as being associated with indicators of lipolysis *in vivo*. Thus, strategies directed at the regulation of phosphorylated perilipin may represent a novel approach to alleviating problems associated with long-term or extreme energy mobilization from adipose tissue.

## 2.6 REFERENCES

- Accorsi, P., N. Govoni, R. Gaiani, C. Pezzi, E. Seren and C. Tamanini. 2005. Leptin, GH, PRL, insulin and metabolic parameters throughout the dry period and lactation in dairy cows. *Reprod. Dom. Anim.* 40:217-223
- Appuhamy, J., B. Cassell, C. Dechow and J. Cole. 2007. Phenotypic relationships of common health disorders in dairy cows to lactation persistency estimated from daily milk weights. *J. Dairy Sci.* 90:4424-4434.
- Arvidsson, E., L. Blomqvist and M. Rydén. 2004. Depot-specific differences in perilipin mRNA but not protein expression in obesity. *J. Intern. Med.* 255(5):595-601.
- Banos, G., M. Coffey, E. Wall and S. Brotherstone. 2006. Genetic relationship between first-lactation body energy and later-life udder health in dairy cattle. *J. Dairy Sci.* 89:2222-2232.
- Brasaemle, D., B. Rubin, I. Harten, J. Gruia-Gray, A. Kimmel and C. Londos. 2000. Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. *J. Bio. Chem.* 275:38486-38493.
- Brasaemle, D., G. Dolios, L. Shapiro and R. Wang. 2004. Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. *J. Bio. Chem.* 279:46835-46842.
- Carmen G., S. and Víctor. 2006. Signalling mechanisms regulating lipolysis. *Cell. Signal.* 18:401-408.
- Clark, J., D. Beede, R. Erdman, J. Goff, R. Grummer, J. Linn, A. Pell, C. Schwab, T. Tomkins, G. Varga and W. Weiss. 2001. National requirements of dairy cattle. Seventh ed. Natl. Acad. Press, Washington D.C.
- Clifford G., D. McCormick, C. Londos, R. Vernon and S. Yeaman. 1998. Dephosphorylation of perilipin by protein phosphatases present in rat adipocytes. *FEBS Letters* 435:125-129.
- Dechow, C., G. Rogers, L. Klei, T. Lawlor and P. VanRaden. 2004. Body condition scores and dairy form evaluations as indicators of days open in US Holsteins. *J. Dairy Sci.* 87:3534-3541.

- Elanco Animal Health. 1996. Body conditioning scoring of dairy cattle.  
<http://dairynutrient.wisc.edu/images/28/01-BCS-Elanco-Handout.pdf>  
 Greenfield, IN.
- Fricke, K., A. Heitland and E. Moronde. 2004. Cooperative activation of lipolysis by protein kinase A and protein kinase C pathways in 3T3-L1 adipocytes. *Endocrinology* 145:4940-4947.
- Granneman, J., H.-P. Moore, R. Granneman, A. Greenberg, M. Obin and Z. Zhu. 2007. Analysis of lipolytic protein trafficking and interactions in adipocytes. *J. Bio. Chem.* 262:5726-5735.
- He, J., H. Jiang, J. Tansey, C. Tang, S. Pu and G. Xu. 2006. Calyculin and okadaic acid promotes perilipin phosphorylation and increase lipolysis in primary rat adipocytes. *Biochim. Biophys. Acta.* 1761:247-255.
- Jaster, E. and T. Wegner. 1981. Beta-adrenergic receptor involvement in lipolysis of dairy cattle subcutaneous adipose tissue during dry and lactating state. *J. Dairy Sci.* 64: 1655-1663.
- Kenward, M. G. and J. H. Roger. 1997. Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics* 53(3): 983-987.
- Kern, P., G. Di Gregorio, T. Lu, N. Rassouli and G. Ranganathan. 2004. Perilipin expression in human adipose tissue is elevated with obesity. *J. Clin. Endocrinol. Metab.* 89(3): 1352-1358.
- Martinez-Botas, J., J. Anderson, D. Tessier, A. Lapillonne, B. Chang, M. Quast, D. Gorenstein, K.-H. Chen and L. Chan. 2000. Absence of perilipin results in leanness and reverses obesity of *Lepr<sup>db/db</sup>* mice. *Nat. Genet.* 26 474-479.
- Miyoshi, H., J. Perfield, S. Souza, W. Shen, H. Zhang, Z. Stancheva, F., Kraemer, M. Obin and A. Greenberg. 2007. Control of adipose triglyceride lipase action by serine 517 of perilipin A globally regulates protein kinase A-stimulated lipolysis of adipocytes. *J. Bio. Chem.* 282:996-1002.
- McNamara, J. 1988. Regulation of bovine adipose tissue metabolism during lactation. 4. Dose-responsiveness to epinephrine as altered by stage of lactation. *J. Dairy Sci.* 71 (3):643-649.

- Patton, J., D. Kenny, S. McNamara, J. Mee, F. O'Mara, M. Diskin and J. Murphy. 2007. Relationships among milk production, energy balance, plasma analytes, and reproduction in Holstein-Friesian cows. *J. Dairy Sci.* 90:649-658.
- Rydén, M., J. Jocken, V. van Harmelen, A. Dicker, J. Hoffstedt, M. Wirén, L. Blomqvist, A. Mairal, D. Langin, E. Blaak and P. Arner. 2007. Comparative studies of the role of hormone-sensitive lipase and adipose triglyceride lipase in human fat cell lipolysis. *Am. J. Physiol. Endocrinol. Metab.* 292(6):E1847-55.
- SAS Institute. 1999. SAS/STAT User's Guide. Version 6.12. SAS Institute Inc., Cary, NC.
- Sumner, J. and J. McNamara. 2007. Expression of lipolytic genes in the adipose tissue of pregnant and lactating Holstein dairy cattle. *J. Dairy Sci.* 90:5237-5246.
- Tansey, J., A. Huml, R. Vogt, K. Davis, J. Jones, K. Fraser, D. Brasaemle, A. Kimmel and C. Londos. 2003. Functional studies on native and mutated forms of perilipins: A role in protein kinase A-mediated lipolysis of triacylglycerols in Chinese hamster ovary cells. *J. Bio. Chem.* 278 (10): 8401-8406.
- Tansey, J., C. Sztalryd, J. Gruia-Gray, D. Roush, J. Zee, O. Gavrilova, M. Reitman, C.-X. Deng, C. Li, A. Kimmel and C. Londos. 2001. Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. *Proc. Natl. Acad. Sci.* 98 (11) 6494-6499.
- Van Arendonk, J., G. Nieuwof, H. Vos and S. Korver. 1991. Genetic aspects of feed intake and efficiency in lactation dairy heifers. *Livest. Prod. Sci.* 29:263-275.
- Veerkamp, R., J. Oldenbroek, H. Van Der Gaast and Van Der Werf, J. 2000. Genetic correlation between days until start of luteal activity and milk yield, energy balance, and live weights. *J. Dairy Sci.* 83:577-583.

- Wang, Y., S. Sullivan, M. Trujillo, M. Lee, S. Schneider, R. Brolin, Y. Kang, Y. Werber, A. Greenberg and S. Fried. 2003. Perilipin expression in human adipose tissues: effects of severe obesity, gender, and depot. *Obes. Res* 11:930-936.
- Zimmermann, R., J. Strauss, G. Haemmerle, G. Schoiswohl, R. Birner-Gruenberger, M. Reiderer, A. Lass, G. Neuberger, F. Eisenhaber, A. Hermetter and R. Zechner. 2004. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* 306:1383-1386



Figure 1: *Beta adrenergic receptor mRNA abundance expressed as fold change differences*. Data were analyzed as log of the starting copy number as described in the text. Resulting least-squares means were transformed to the linear scale for calculation of fold change relative to mid lactation.

\*  $P < 0.05$

Figure 2: *Abundance of HSL mRNA and protein in early (5-14 DIM) and mid (176-206 DIM) lactation*. **A.** Representation of the fold change of HSL mRNA abundance, relative to mid lactation. **B.** A representative western blot using HSL antibody, showing two cows in early (lanes 1 and 3) and two cows in mid (lanes 2 and 4) lactation. **C.** Abundance of HSL protein, as determined by quantification of western blots **D.** Representative western blot using phosphoHSL antibody, showing the same cows as in 2B. **E.** Abundance of serine 563 phosphorylated HSL, as determined by quantification of western blots. *Solid bars represent early lactation and open bars represent mid lactation.*

\*  $P < 0.05$

Figure 3: *Abundance of perilipin mRNA and protein in early (5-14 DIM) and mid (176-206 DIM) lactation*. **A.** Representation of the fold change of perilipin mRNA abundance, relative to mid lactation. **B.** A representative western blot using perilipin antibody, showing two cows in early (lanes 1 and 3) and two cows in mid (lanes 2 and 4) lactation. **C.** Abundance of perilipin protein, as determined by quantification of western blots **D.** Representative western blot using phosphor-

(Ser/Thr) PKA substrate antibody, showing the same cows as in 3B. **E.** Abundance of serine-threonine phosphorylated perilipin, as determined by quantification of western blots. *Solid bars represent early lactation and open bars represent mid lactation.*

\*  $P < 0.05$

Figure 4: *Verification of Phosphorylated (Ser/Thr) PKA Substrate antibody for detection of phosphorylation of perilipin.* A membrane was exposed to Phospho (Ser/Thr) PKA substrate primary antibody and imaged, as described in the text. **(A).** The same membrane was stripped, then exposed to perilipin antibody (B). Lane 1: biotinylated protein ladder. Lanes 2-5: Adipose tissue protein samples from a four different cows.

Figure 5: *Regulation of Lipolysis.* Under stimulated conditions (shaded arrows and proteins), catecholamines bind with  $\beta$ -AR to activate adenylyl cyclase and convert ATP to cAMP. This conversion allows for activation of PKA. PKA phosphorylates cytosolic HSL and lipid bound perilipin, facilitating translocation of HSL to the lipid droplet and interaction of HSL with triacylglycerols. During basal lipolysis (open arrows and unshaded proteins), PKA has minimal activity and phosphorylation of HSL and perilipin are reduced. Unphosphorylated perilipin is thought to act as a barrier, hindering access of HSL to the lipid droplet.

Figure 1

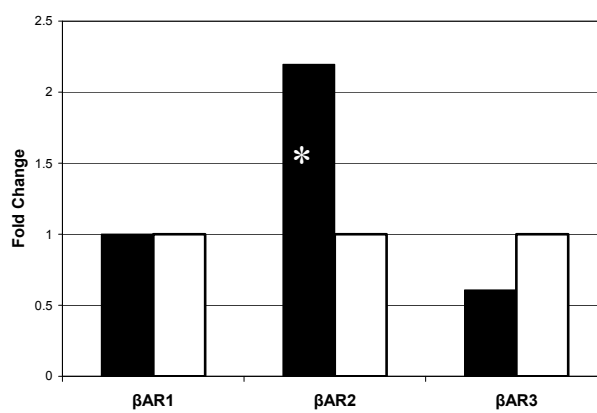


Figure 2

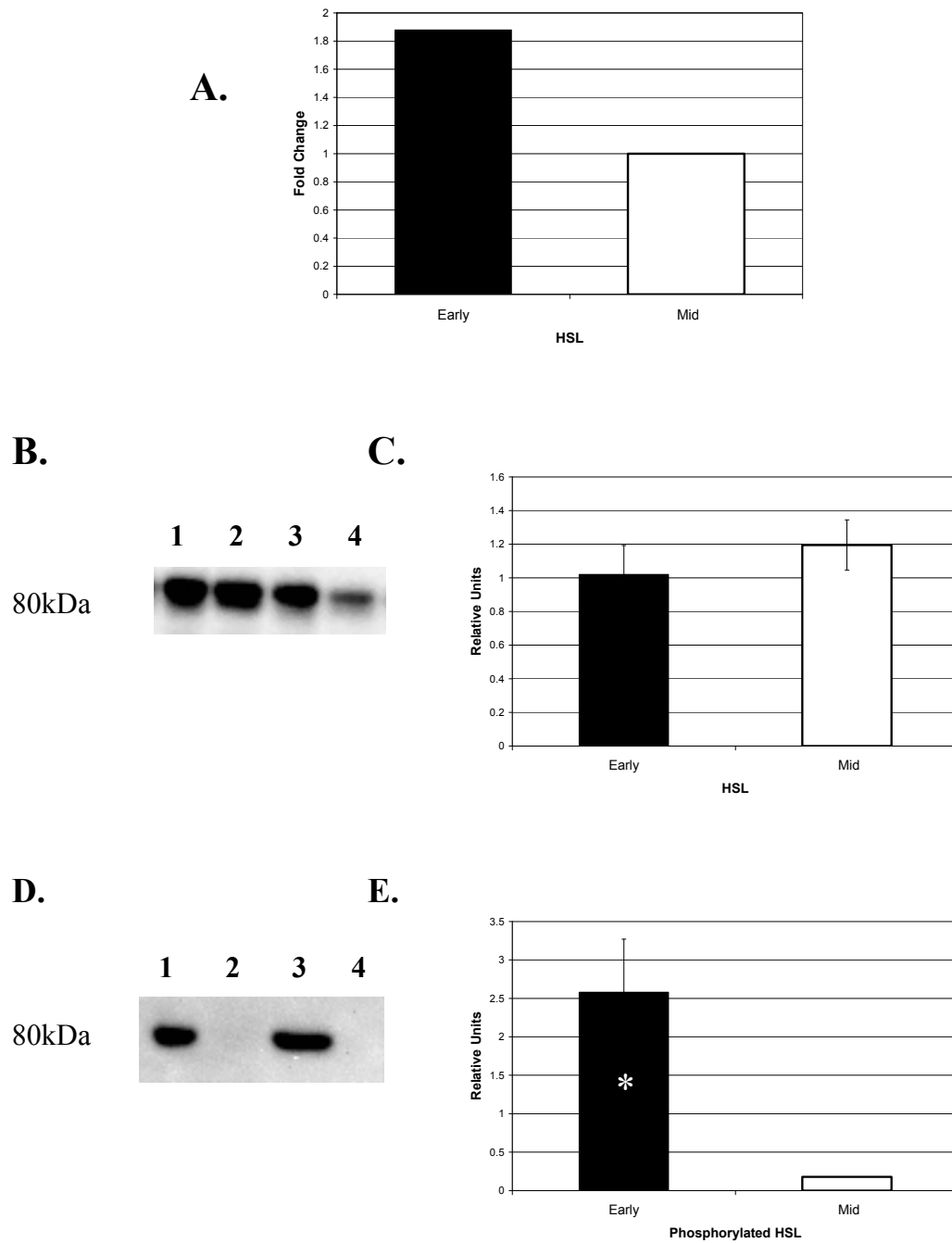


Figure 3

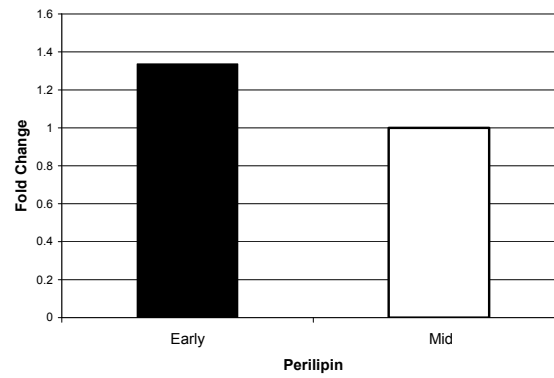
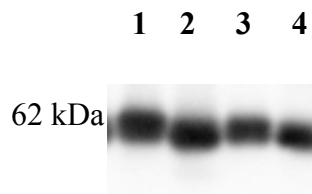
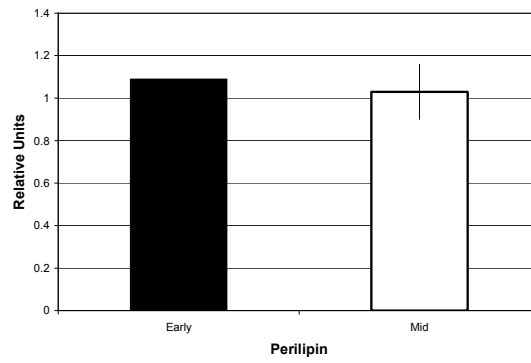
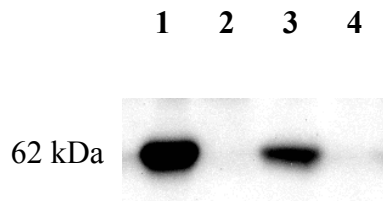
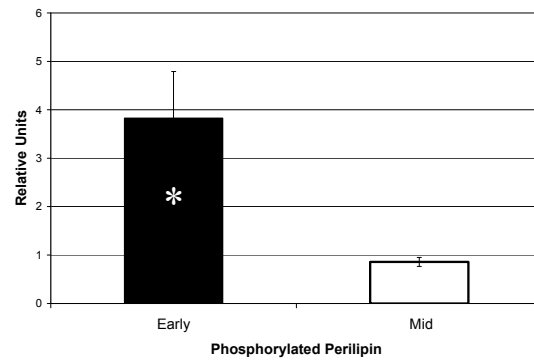
**A.****B.****C.****D.****E.**

Figure 4

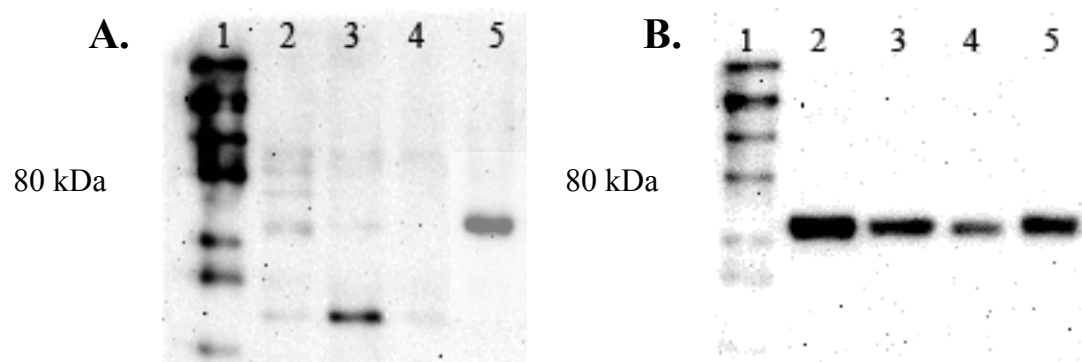


Figure 5

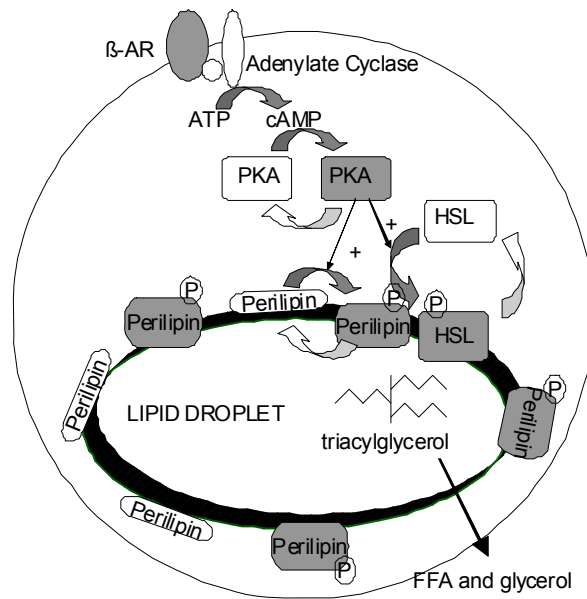


Table 1: Antibody Concentrations and Corresponding Amount of Protein.

Antibody	Loading Quantity*	1° antibody Concentration**
<i>Perilipin</i> <sup>1</sup>	10 µg	1:1000
<i>Phospho (Ser/Thr) PKA Substrate</i> <sup>2</sup>	400 µg	1:250
<i>Hormone Sensitive Lipase</i> <sup>2</sup>	100 µg	1:1000
<i>Phospho-Hormone Sensitive Lipase (Ser563)</i> <sup>2</sup>	400 µg	1:1000

<sup>1</sup> antibodies from Chemicon

<sup>2</sup> antibodies from Cell Signaling

\* Amount of protein mixed with sample buffer

\*\* ratio of antibody to TBS-T in 5% dry milk



Table 2: Primer Sequences for Perilipin, Hormone Sensitive Lipase, Beta Adrenergic Receptors, and 60S Ribosomal Protein 32L for PCR, Plasmid and Gene Specific Primer cDNA Synthesis.

Gene	Primer Sequence
Perilipin	forward – 5'-GAC ACT GCC GAG TAT GCT GC-3' reverse -- 5'-AAG ACC CGT AGG ACC TCA GG-3'
HSL <sup>a</sup>	forward – 5'-ATC TTC TTT CGC ACC AGC CAC-3' reverse – 5'-AGG TGT GAA CTG GAA ACC-3'
$\beta$ -AR <sub>1</sub>	forward --5'-AGC AGA AGG CAC TCA AGA CG-3' reverse – 5' GTT GAA GAA GAC GAA GAG GC-3'
$\beta$ -AR <sub>2</sub>	forward – 5'-AGA TCG ACA AAT CTG AGG GC-3' reverse– 5'-AA GTG CCC ATG ATA ATG CC-3'
$\beta$ -AR <sub>3</sub>	forward – 5'-GCT GAT GCT CTT CGT CTA CG-3' reverse– 5'-CCA CAA AGA AAG GCA ACC AG-3'
RPL	forward – 5' –TCT GGC CCT TGA ATC TTC TG-3' reverse– 5'-CCT CGT GAA GCC TAA CAT GC-3'

<sup>a</sup>Abbreviations: hormone sensitive lipase, HSL; beta adrenergic receptor 1,  $\beta$ AR<sub>1</sub>; beta adrenergic receptor 2,  $\beta$ AR<sub>2</sub>; beta adrenergic receptor 3,  $\beta$ AR<sub>3</sub>; 60S ribosomal protein 32L, RPL

Table 3: Least-Squares Means (SE) of Cows in Early (5-14 DIM, n = 11) and Mid (176-208 DIM, n = 9) Lactation for Production Traits, Abundance of mRNA, Protein, and Phosphorylated Protein, and *in vivo* Indicators of Lipolysis.

	Early Lactation	Mid Lactation	Unit of Measurement	P-value
Milk Yield <sup>a</sup>	36.7 (2.2)	36.1 (4.82)	kg	0.863
ME MY <sup>b</sup>	14,902 (370)	12,245 (410)	kg	<0.001
ME Pro	439 (14)	377 (15)	kg	0.008
ME Fat	530 (25)	442 (28)	kg	0.029
$\beta$ AR <sub>1</sub> mRNA	2.454 (0.110)	2.632 (0.121)	LSCN <sup>c</sup>	0.176
$\beta$ AR <sub>2</sub> mRNA	4.443 (0.102)	4.075 (0.108)	LSCN	0.025
$\beta$ AR <sub>3</sub> mRNA	2.247 (0.038)	2.158 (0.038)	LSCN	0.134
HSL mRNA	5.319 (0.127)	5.075 (0.135)	LSCN	0.192
PLIN mRNA	5.405 (0.150)	5.352 (0.157)	LSCN	0.814
HSL	-0.12 (0.18)	-0.04 (0.19)	log <sub>e</sub> (Relative units)	0.734
PHSL	2.62 (0.705) <sup>d</sup>	-	Relative units	0.001 <sup>e</sup>
PLIN	-0.05 (0.11)	-0.10 (0.12)	log <sub>e</sub> (Relative units)	0.742
PPLIN	-1.02 (0.20)	-0.25 (0.23)	log <sub>e</sub> (Relative units)	0.0006
NEFA	6.91 (0.11)	4.83 (0.12)	log <sub>e</sub> ( $\mu$ Eq/L based on oleic acid)	<0.0001
GLYC	4.42 (0.08)	3.65 (0.08)	log <sub>e</sub> ( $\mu$ M)	<0.0001

<sup>a</sup>Abbreviations: Milk Yield, average daily milk production from two days before through two days after the biopsy; ME MY, mature equivalent milk yield; ME Pro, mature equivalent protein; ME Fat, mature equivalent fat;  $\beta$ AR, beta-adrenergic receptor; PLIN, perilipin; PHSL, phosphorylated hormone sensitive lipase; PPLIN, phosphorylated perilipin; NEFA, non-esterified fatty acid; GLYC, glycerol

<sup>b</sup>Mature equivalence values were predicted following 270 to 305 DIM, and the average DIM was similar between early and mid lactation groups (P = 0.726)

<sup>c</sup>log of the starting copy number

<sup>d</sup>PHSL data were not evaluated using mixed models, because it was only detected in a single mid lactation cow. The raw mean and standard error of early lactation cows are presented.

<sup>e</sup>p-value represents a Chi-square test based on the presence or absence of phosphorylated HSL

Table 4: Correlation Coefficients (r) from Cows in Early Lactation (5-14 DIM, n = 11) for the Abundance and Phosphorylation of Proteins Involved in Lipolysis, and *in vivo* Indicators of Lipolysis.

	PPLIN	HSL	PHSL	NEFA	GLYC
PLIN	0.51	0.17	0.03	-0.22	0.11
PPLIN		0.73*	0.51	0.60*	0.73*
HSL			0.63*	0.65*	0.65*
PHSL				0.61	0.73*
NEFA					0.80**

\*P<0.05

\*\*P<0.01

Table 5: Correlation Coefficients (r) from Cows in Mid Lactation (176-206 DIM, n = 9) for the Abundance and Phosphorylation of Proteins Involved in Lipolysis, and *in vivo* Indicators of Lipolysis.

	PPLIN	HSL	NEFA	GLYC
PLIN	0.09	0.52	0.06	0.42
PPLIN		0.31	0.50	0.65 <sup>+</sup>
HSL			0.02	0.75*
NEFA				0.39

\*P<0.05

<sup>+</sup>P=0.06

# **CHAPTER 3. ADIPOSE TRIGLYCERIDE LIPASE ABUNDANCE IS INCREASED DURING TIMES OF LOW ENERGY MOBLIZATION IN HOLSTEIN COWS**

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## **3.1 ABSTRACT**

The novel lipase adipose triglyceride lipase (ATGL) and its activator, comparative gene identity-58 (CGI-58), were studied due to their association with lipolysis in rodents. In rodent models, upon activation phosphorylation of perilipin, CGI-58 translocates from the lipid droplet to interact with ATGL in the cytosol. The CGI-58/ATGL complex then translocates to the lipid droplet where ATGL is thought to initialize the catabolism of triacylglycerols by hydrolyzing triacylglycerols to diacylglycerols. Thus, we hypothesized ATGL and CGI-58 abundance would increase during early lactation to accompany increased lipolytic activity. Semi-quantitative western blotting was used to detect protein abundance from early (5-14 days in milk; n=11) and mid (176-206 days in milk; n=9) lactation cows. Serum concentrations of non-esterified fatty acids and glycerol were measured lipolytic indicators. Both non-esterified fatty acids and glycerol significantly increased in early compared to mid lactation cows. However, in contrast to our hypothesis, ATGL abundance increased in mid lactation cows compared to early lactation cows,

indicating increased abundance of ATGL during times of low lipolytic activity. CGI-58 abundance was similar between early and mid lactation cows. Neither ATGL nor CGI-58 was significantly correlated with lipolytic indicators during early or mid lactation. Although more research is needed to fully understand the role of these novel proteins in lipolysis in lactation dairy cows, the increase in ATGL abundance in mid lactation supports its putative role as a regulator of basal lipolysis.

### 3.2 INTRODUCTION

Milk production increased 6,174 kg in cows born in 2006 compared to cows born in 1957 (AIPL, 2008). Energy requirements during early lactation often cannot be met by energy intake (Van Arendonk, 1991), resulting negative energy balance. Cows that experience severe or prolonged negative energy balance are at risk for decreased fitness traits such as, decreased reproductive efficiency (Lopez et al., 2005; Patton et al., 2007; Veerkamp et al., 2000), higher somatic cell counts in subsequent lactations (Banos et al., 2006), increased risk of metabolic disorders and mastitis (Appuhamy et al., 2007; Dechow et al., 2004) and increased risk of laminitis (Collard et al., 2000).

To meet energy requirements for milk production during early lactation, energy substrates in adipose tissue are mobilized via lipolysis. The traditional view of triacylglyceride catabolism in adipose tissue is that catecholamines bind to  $\beta$ -adrenergic receptors to activate adenylyl cyclase via G-proteins. Adenylyl cyclase increases the cyclic adenosine monophosphate (Campagna et al., 2008) to ATP ratio, resulting in the activation protein kinase A (**PKA**) which phosphorylates hormone sensitive lipase (**HSL**). Upon phosphorylation, HSL translocates from the

cytosol to the lipid droplet where it interacts with its lipid substrate, and hydrolyzes triacylglycerides to free fatty acids and glycerol. This traditional view of triacylglyceride catabolism has been expanded with the discovery of additional proteins. Perilipin is a protein that coats the lipid droplet and prevents hydrolysis of triacylglycerides by blocking access of HSL to triacylglycerides. However, upon phosphorylation of perilipin by PKA, phosphorylated perilipin interacts with phosphorylated HSL to enhance lipolysis (Granneman et al., 2007). More recently, two additional proteins that influence triacylglyceride catabolism have been identified: adipose triglyceride lipase (ATGL) and comparative gene identity-58 (CGI-58).

Adipose triglyceride lipase was discovered when mice lacking HSL were found to be lean with an accumulation of diacylglycerides (Zimmermann et al., 2004). Since this initial discovery, ATGL has been shown to hydrolyze triacylglycerides to diacylglycerides, potentially initiating triacylglyceride catabolism (Schweiger et al., 2006). Additional research supports ATGL working in tandem with HSL, based on correlation of ATGL and HSL expression (Steinberg et al., 2007) and similar expression pattern of HSL and ATGL by multiple stimuli (Kralisch et al., 2005). Adipose triglyceride lipase may also have a role in preventing irregular adipogenesis, as increased adiposity and adipose tissue accumulation in muscle and cardiac tissue are observed in patients with the rare genetic disease called Neutral Lipid Storage Disease, which is caused by mutations in ATGL (Akiyama et al., 2007; Haemmerle et al., 2006).



CGI-58 has been suggested as a regulator of ATGL (Lass et al., 2006; Schweiger et al., 2006). CGI-58 interacts with perilipin and adipocyte differentiation-related protein at the lipid droplet (Subramanian et al., 2004; Yamaguchi et al., 2004; Yamaguchi et al., 2007). Upon phosphorylation of perilipin, CGI-58 translocates from the lipid droplet to the cytosol where it interacts with ATGL (Granneman et al., 2007; Lass et al., 2006; Schweiger et al., 2006), increasing triglyceride hydrolase activity of ATGL (Lass et al., 2006; Schweiger et al., 2006). When CGI-58 is not present, adipocytes exhibit decreased basal and stimulated lipolysis (Yamaguchi et al., 2007). Interestingly, CGI-58 exclusively interacts with ATGL and not HSL (Lass et al., 2006), suggesting CGI-58 is a regulator of ATGL activity.

As an extension of previous work in which phosphorylation of perilipin was associated with lipolytic indicators, the first objective of this study was to determine if the abundance of ATGL and CGI-58 significantly differs between early and mid lactation cows undergoing different rates of lipolysis. Based on existing literature, it is expected that ATGL abundance will be greater in early lactation cows when lipolysis is increased. As a regulator of ATGL, it is also expected that CGI-58 will be more abundant in early lactation cows. Therefore, the second objective of this study was to determine if the abundance of ATGL and CGI-58 are associated indicators of lipolysis during early and mid lactation.

### **3.3 MATERIALS AND METHODS**

#### **3.3.1 Animals**

Animals of this study were described previously (Chapter 2). Briefly, adipose tissue was collected from early (n=11; 5-14 DIM) and mid (n=9; 176-206 DIM)

lactation Holstein cows between May and October 2007. One early and one mid lactation cow were sampled each day biopsies were collected, with the exception of 3 days when a single cow was biopsied. Approximately five grams of adipose tissue was collected under local anesthesia and immediately snap-frozen in liquid nitrogen until stored at  $-80^{\circ}\text{C}$ . A blood sample was collected following the biopsy via jugular venipuncture and serum was stored at  $-20^{\circ}\text{C}$ .

Animals ranged in body condition scores from 2.75 to 4.5 on a 5-point scale (Elanco, 1996, A18478), and were in their second to fifth lactation. Milk production data from monthly DHIA test-day sampling were evaluated. All cows were free of mastitis, severe lameness, and other major health problems. The protocol for this experiment was approved by the Iowa State University Institutional Animal Care and Use Committee.

### **3.3.2 Serum Assays**

Serum was assayed for glycerol using Free Glycerol Reagent (Sigma, F6428) and for NEFA using the NEFA-C kit (Wako, 999-75406), both according to manufacturer's protocol. All samples were assayed in triplicate using a 96-well plate format, and data absorbencies were read on Tecan Spectrafluor Plus (Tecan Group Ltd).

### 3.3.3 Semi-quantitative Western Blotting

Protein was extracted using a procedure that dissociates the lipid droplet (Wang et al., 2003) and stored at -80°C. Protein concentrations were measured using BCA Protein Assay kit (Pierce, 23227) with absorbencies read on Tecan Spectrafluor Plus (Tecan Group Ltd.).

Semi-quantitative western blotting was used to measure ATGL and CGI-58 protein abundance. Equal protein quantities (400 µg or 1000 µg for ATGL or CGI-58, respectively) of all samples (N=20) were run together on a single gel, and quantified relative to an additional sample that was chosen at random. Quantification of all samples was replicated across two gels. Proteins were separated on 8% or 12% SDS-PAGE gels for detection of ATGL and CGI-58, respectively, using commercially available antibodies (Cell Signaling and Everest Biotech, respectively). A biotinylated protein marker (Cell Signaling, 7727) was utilized for size verification of CGI-58. Gels were transferred to nitrocellulose membranes using a semi-dry transfer unit (Amersham, TE 70). Membranes were blocked in 5% dry milk in Tris buffered saline with Tween-20 (TBS-T). Primary antibodies were exposed to membranes overnight at 4°C and then thrice rinsed with TBS-T. Membranes were exposed to Horseradish Peroxidase (**HRP**)-conjugated secondary antibody (GE Healthcare, NA934A or Santa Cruz, SC-2768, for ATGL and CGI-58, respectively) for 2 hours at room temperature. After membranes were rinsed with TBS-T, signals were detected using ECL Plus Western Blotting Detection Reagents (Amersham, RPN 2132), and resulting signal was captured and quantified using an Alpha Imager Gel Documentation System and accompanying software

(Alpha Innotech). Protein quantification for each cow was measured in duplicate, and the duplicate measures were utilized in statistical analyses.

### **3.3.4 Statistical Analysis**

Protein differences between early and mid lactation were analyzed using PROC MIXED in SAS (SAS, 1999). The mixed model used for analysis included stage of lactation as a fixed effect, and cow and day of biopsy (1-5) as random effects. The method described by Kenward and Rogers (1997) was used to determine denominator degrees of freedom. Correlations between average protein abundance for each cows, and average NEFA and glycerol for each cow were determined using PROC CORR in SAS (SAS, 1999) within stage of lactation. Values of ATGL and CGI-58 were also correlated with phosphorylated perilipin, phosphorylated HSL, perilipin, and HSL previously measured from the same adipose tissue samples (Chapter 2). Correlations involving phosphorylated HSL were only examined for cows in early lactation, since phosphorylated HSL was not detected in mid lactation cows.

## **3.4 RESULTS**

A summary of the animals is given in Chapter 2. Briefly, early and mid lactation cows were similar ( $P>0.05$ ) in body condition score, number of lactations and milk production at the time of biopsy. However, early lactation cows had significantly greater 305d mature equivalence (ME) milk ( $P<0.001$ ), fat ( $P=0.029$ ) and protein ( $P=0.008$ ) production in the lactation they were biopsied. As previously determined, NEFA and glycerol were greater ( $P<0.001$ ) in early compared to mid

lactation cows, confirming that adipose tissue was being mobilized at a significantly higher rate in early compared to mid lactation cows.

Adipose triglyceride lipase abundance mimics HSL abundance in rodents, but has not yet been investigated in cattle. Semi-quantitative western blotting revealed that bovine ATGL protein abundance was significantly greater in mid versus early lactation cows ( $P=0.04$ ; Figure 1). However, ATGL protein abundance was not significantly correlated with lipolytic indicators or with HSL or perilipin.

CGI-58 interacts with ATGL in the cytosol, and might act as a regulator of ATGL (Granneman et al., 2007). The expected size of bovine CGI-58 is 39 kDa, which is smaller than the primary band (~50 kDa) observed (Figure 2 A). However, a secondary band of the expected size of 39 kDa was also present, suggesting bovine CGI-58 may undergo post-translational modification. For this research, the primary band of ~50 kDa was quantified. The abundance of CGI-58 did not significantly differ ( $P=0.96$ ) between early and mid lactation cows (Figure 2 B and C). Abundance of CGI-58 and ATGL abundance were correlated in early ( $P=0.03$ ), but not in mid lactation ( $P=0.99$  See Table 2).

### **3.5 DISCUSSION**

Adipose tissue mobilization during the transition period has been associated with increased incidences of disease (mastitis, milk fever, and ketosis), and these diseases have been estimated to cost the United States dairy industry approximately \$2.6 billion (Sonstegard, 2003). Additionally, losses due to involuntary culling resulting from poor reproductive fitness and udder longevity are not included in this

large estimated cost to the dairy industry. Thus, a greater understanding of adipose tissue mobilization and its relation to health and fitness traits is needed.

Previous work from this lab has demonstrated that phosphorylation of perilipin and HSL are associated with lipolytic indicators from early lactation cows (Chapter 2). Additionally, phosphorylation of perilipin and protein abundance of HSL remains significantly correlated with lipolytic indicators during mid lactation. The novel lipase, ATGL, and its regulator, CGI-58, are evaluated in this study as an extension of our previous work with novel proteins involved in lipolysis. To our knowledge, this is the first study to investigate ATGL and CGI-58 in ruminants.

In contrast to our initial hypothesis, ATGL abundance was significantly greater in mid lactation when lipolysis was minimal. Our results are more reflective of ATGL acting as a steady state lipase, or basal lipase. At this time limited literature exists exploring the relationship of ATGL abundance or activity with basal lipolysis. Blunted lipolytic activity in human adipocytes with low or ablated ATGL expression under basal conditions indicates that ATGL may act as a basal lipase (Mairal et al., 2006; Ryden et al., 2007). However, lipolytic activity remained unaffected in stimulated human adipocytes (Ryden et al., 2007). These results suggest that although human ATGL contributes to basal lipolysis, it may not significantly contribute under stimulated conditions which in part could be due to increased HSL phosphorylation. A more recent study using mouse embryonic fibroblasts (**MEF**) observed a reduction in adipocyte size in cells overexpressing ATGL during unstimulated conditions (Miyoshi et al., 2008). However, at this time, 3T3-L1 adipocyte cell line studies have not indicated ATGL as a basal lipase

implying the difficulty of simulating basal *in vivo* conditions. With study of basal lipolysis accounting for the varying metabolic states of individuals, this is a very difficult challenge to overcome. However, the use of rodent models might be useful in determining factors that contribute to basal lipolysis.

As a regulator of ATGL, CGI-58 abundance could provide insight in the role of both ATGL and CGI-58 during times of lipolytic activity. From this study, CGI-58 abundance did not differ between stages of lactation. As little literature relating to CGI-58 abundance outside of Neutral Lipid Storage disease exists, it is difficult to predict the role CGI-58 abundance in dairy cattle. Cultured adipocytes have demonstrated that CGI-58 interacts with perilipin and will translocate to the cytosol upon phosphorylation of perilipin (Granneman et al., 2007), where it then interacts with ATGL (Subramanian et al., 2004; Yamaguchi et al., 2006). In this study, CGI-58 abundance is not correlated with phosphorylation of perilipin or HSL or other lipolytic indicators, and its abundance is similar in early and mid lactation. However, these results do not preclude the possibility that the translocation or localization of CGI-58 within the cell plays a critical role in regulation lipolysis.

### **3.6 CONCLUSION**

Our results implicate ATGL as a novel protein associated with the physiological changes in adipose tissue from early to mid lactation. Because ATGL abundance increased in mid lactation when lipolysis decreased, these results highlight the complexity of lipid metabolism in adipose tissue throughout a lactation cycle. One potential explanation for this result is that the primary role for ATGL is in the regulation of lipolysis during times of neutral or positive energy balance, whereas

HSL is activated in response to negative energy balance in early lactation. Our results did not detect differences in CGI-58 abundance according to stage of lactation, but its cellular localization still needs to be considered carefully. Together, these results demonstrate the presence and potential importance of novel proteins in the regulation of lipolysis throughout lactation. Further research is needed to clarify the complexities of this system that include potential regulation at the level of mRNA and protein abundance, phosphorylation and sub-cellular localization.

### **3.7 REFERENCES**

- AIPL. 2008. Trend in milk bv for holstein or red and white No. 2008. Animal Improvement Programs Laboratory.
- Akiyama, M. et al. 2007. Novel duplication mutation in the patatin domain of adipose triglyceride lipase (pnpla2) in neutral lipid storage disease with severe myopathy. *Muscle Nerve* 36: 856-859.
- Appuhamy, J. A., B. G. Cassell, C. D. Dechow, and J. B. Cole. 2007. Phenotypic relationships of common health disorders in dairy cows to lactation persistency estimated from daily milk weights. *J Dairy Sci* 90: 4424-4434.
- Banos, G., M. P. Coffey, E. Wall, and S. Brotherstone. 2006. Genetic relationship between first-lactation body energy and later-life udder health in dairy cattle. *J Dairy Sci* 89: 2222-2232.
- Campagna, F. et al. 2008. Novel mutations in the adipose triglyceride lipase gene causing neutral lipid storage disease with myopathy. *Biochem Biophys Res Commun*.
- Collard, B. L., P. J. Boettcher, J. C. Dekkers, D. Petitclerc, and L. R. Schaeffer. 2000. Relationships between energy balance and health traits of dairy cattle in early lactation. *J Dairy Sci* 83: 2683-2690.
- Dechow, C. D., G. W. Rogers, L. Klei, T. J. Lawlor, and P. M. VanRaden. 2004. Body condition scores and dairy form evaluations as indicators of days open in us holsteins. *J Dairy Sci* 87: 3534-3541.



- Elanco (Editor), 1996. Body condition scoring in dairy cattle, AI 8478.
- Granneman, J. G. et al. 2007. Analysis of lipolytic protein trafficking and interactions in adipocytes. *J Biol Chem* 282: 5726-5735.
- Haemmerle, G. et al. 2006. Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. *Science* 312: 734-737.
- Kenward, M. G., and J. H. Roger. 1997. Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics* 53: 983-997.
- Kralisch, S. et al. 2005. Isoproterenol, tnfa, and insulin downregulate adipose triglyceride lipase in 3T3-L1 adipocytes. *Mol Cell Endocrinol* 240: 43-49.
- Lass, A. et al. 2006. Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman syndrome. *Cell Metab* 3: 309-319.
- Lopez, H., D. Z. Caraviello, L. D. Satter, P. M. Fricke, and M. C. Wiltbank. 2005. Relationship between level of milk production and multiple ovulations in lactating dairy cows. *J Dairy Sci* 88: 2783-2793.
- Mairal, A., D. Langin, P. Arner, and J. Hoffstedt. 2006. Human adipose triglyceride lipase (PNPLA2) is not regulated by obesity and exhibits low in vitro triglyceride hydrolase activity. *Diabetologia* 49: 1629-1636.
- Miyoshi, H., J. W. Perfield, 2nd, M. S. Obin, and A. S. Greenberg. 2008. Adipose triglyceride lipase regulates basal lipolysis and lipid droplet size in adipocytes. *J Cell Biochem* 105: 1430-1436.
- Patton, J. et al. 2007. Relationships among milk production, energy balance, plasma analytes, and reproduction in Holstein-Friesian cows. *J Dairy Sci* 90: 649-658.
- Ryden, M. et al. 2007. Comparative studies of the role of hormone-sensitive lipase and adipose triglyceride lipase in human fat cell lipolysis. *Am J Physiol Endocrinol Metab* 292: E1847-1855.
- SAS (Editor), 1999. *SAS/STAT User's Guide* version 6.12. SAS Institute Inc., Cary, NC.
- Schweiger, M. et al. 2006. Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism. *J Biol Chem* 281: 40236-40241.

- Sonstegard, T., R. Li and C. Van Tassell. 2003. Identification, validation and fine-mapping of quantitative trait loci in dairy cattle No. 2008.
- Steinberg, G. R., B. E. Kemp, and M. J. Watt. 2007. Adipocyte triglyceride lipase expression in human obesity. *Am J Physiol Endocrinol Metab* 293: E958-964.
- Subramanian, V. et al. 2004. Perilipin a mediates the reversible binding of cgi-58 to lipid droplets in 3t3-l1 adipocytes. *J Biol Chem* 279: 42062-42071.
- Van Arendonk, J., G. Niewof, H. Vos and S. Korver. 1991. Genetic aspects of feed intake and efficiency in lactation dairy heifers. *Livest. Prod. Sci* 29: 263-275.
- Veerkamp, R. F., J. K. Oldenbroek, H. J. Van der Gaast, and J. H. Van der Werf. 2000. Genetic correlation between days until start of luteal activity and milk yield, energy balance, and live weights. *J Dairy Sci* 83: 577-583.
- Wang, Y. et al. 2003. Perilipin expression in human adipose tissues: Effects of severe obesity, gender, and depot. *Obes Res* 11: 930-936.
- Yamaguchi, T., N. Omatsu, S. Matsushita, and T. Osumi. 2004. Cgi-58 interacts with perilipin and is localized to lipid droplets. Possible involvement of cgi-58 mislocalization in chnarin-dorfman syndrome. *J Biol Chem* 279: 30490-30497.
- Yamaguchi, T. et al. 2007. Cgi-58 facilitates lipolysis on lipid droplets but is not involved in the vesiculation of lipid droplets caused by hormonal stimulation. *J Lipid Res* 48: 1078-1089.
- Yamaguchi, T., N. Omatsu, A. Omukae, and T. Osumi. 2006. Analysis of interaction partners for perilipin and adrp on lipid droplets. *Mol Cell Biochem* 284: 167-173.
- Zimmermann, R. et al. 2004. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* 306: 1383-1386.

Figure 1: *Abundance of ATGL protein in early and mid lactation of dairy cattle.* **A.** Abundance of ATGL protein as determined by semi-quantitative western blotting. **B.** Representative western blot of ATGL depicted by two early (lanes 1 and 3) and two mid (lanes 2 and 4) lactation cows. *Solid bars represent early lactation cows and open bars represent mid lactation cows.*

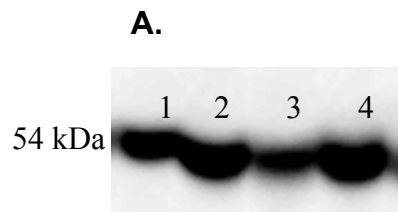
\*  $P < 0.05$

Figure 2: *Abundance of CGI-58 protein in early and mid lactation of dairy cattle.* **A.** Size of bovine CGI-58 was confirmed through western blotting with a biotinylated protein marker on the left side of the western blot. The primary band is located at ~ 50 kDa, and the secondary band at the expected size is located at 39 kDa. **B.** Abundance of CGI-58 protein as determined by semi-quantitative western blotting. **C.** Representative western blot of CGI-58 depicted by two early (lanes 1 and 3) and two mid (lanes 2 and 4) lactation cows. *Solid bars represent early lactation cows and open bars represent mid lactation cows.*

Figure 3: *Proposed basal lipolysis in dairy cattle.* Interpretation of our research and literature suggests that during mid lactation when basal lipolysis is expected to be decreased, resulting in decreased phosphorylation of perilipin by PKA. Limited phosphorylation of perilipin decreases the rate of translocation of CGI-58 (1). Minimal translocated CGI-58 interacts with more abundant ATGL in the cytosol, resulting in CGI-58 and ATGL translocate to small lipid droplets (3). ATGL interacts

with triacylglycerides and hydrolyzes to diacylglycerides (4). It is possible that HSL is activated by many different pathways during basal lipolysis would reduce diacylglycerides to monoacylglycerides and then to free fatty acids (**FFA**) and glycerol. However, during times of neutral and positive energy balance, FFA can be re-etherified by surrounding tissue and not yield accurate NEFA concentrations. Gray filled boxes and arrows indicate inactivity or lessened activity by PKA pathway. Clear filled boxes and arrows indicate increased activity of PKA pathway.

Figure 1



**B.**

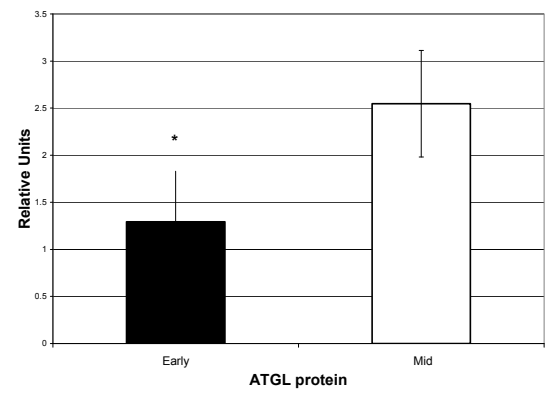


Figure 2

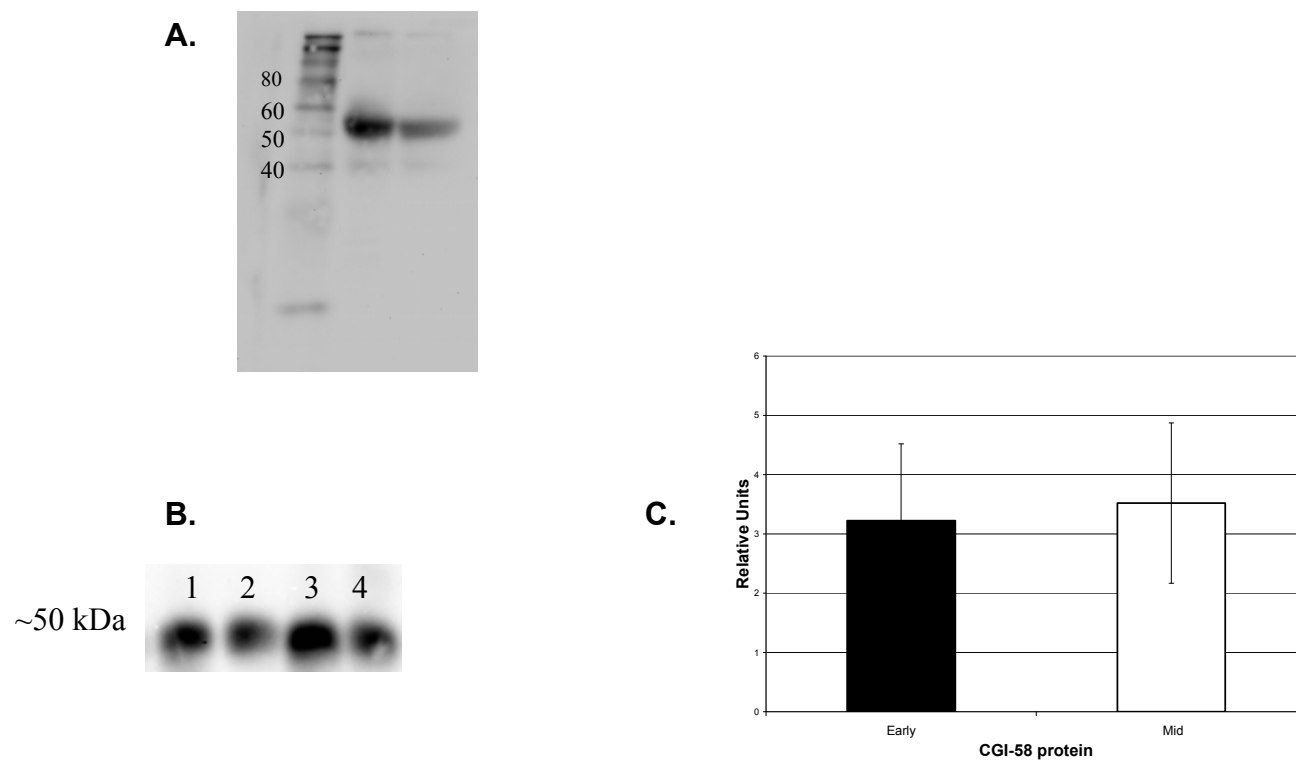


Figure 3

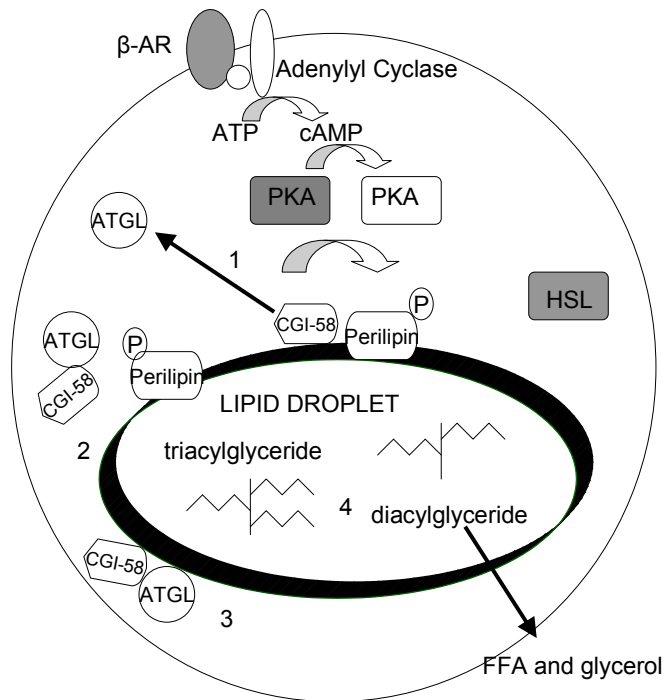


Table 1: Correlation Coefficients (r) of ATGL protein with phosphorylation and protein abundance of HSL and perilipin and lipolytic indicators during both Early (5-14 DIM) and Mid (176-208 DIM) lactation cows.

	Early Lactation N=11 <sup>a</sup>	Mid Lactation N=9
HSL <sup>b</sup>	-0.33	0.41
PHSL	0.30	-- <sup>c</sup>
Plin	-0.37	-0.08
PPlin	-0.28	-0.48
Glycerol	0.05	0.16
NEFA	0.31	-0.47

\*P<0.05

<sup>a</sup> Variation of N is due to number of cows used to detection, protein and lipolytic indicators.

<sup>b</sup> abbreviations: HSL, hormone sensitive lipase; PHSL, phosphorylated hormone sensitive lipase; Plin, perilipin; PPlin, phosphorylated perilipin; NEFA, nonesterified fatty acid

<sup>c</sup>PHSL protein data were not evaluated for mid lactation due to only one cow being detected during mid lactation.



Table 2: Correlation Coefficients (r) of CGI-58 protein with phosphorylation, protein abundance of HSL and perilipin and lipolytic indicators during both Early (5-14 DIM) and Mid (176-208 DIM) lactation cows.

	Early Lactation N=11 <sup>a</sup>	Mid Lactation N=9
HSL <sup>b</sup>	-0.47	-0.03
PHSL	0.04	-- <sup>c</sup>
Plin	0.11	0.37
PPlin	-0.40	-0.53
ATGL	0.64*	0.01
Glycerol	-0.27	-0.16
NEFA	-0.22	0.01

\*P<0.05

<sup>a</sup> Variation of N is due to number of cows used to detection, protein and lipolytic indicators.

<sup>b</sup> abbreviations: HSL, hormone sensitive lipase; PHSL, phosphorylated hormone sensitive lipase; Plin, perilipin; PPlin, phosphorylated perilipin; ATGL, adipose triglyceride lipase; NEFA, nonesterified fatty acid; CGI-58, comparative gene identity-58

<sup>c</sup>PHSL protein data were not evaluated for mid lactation due to only one cow being detected during mid lactation.

## **CHAPTER 4. LAG BETWEEN LIPOLYTIC INDICATORS AND ADIPOCYTE SIZE AND BETA-ADRENERGIC SENSITIVITY IN EARLY AND MID LACTATION COWS**

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### **4.1 INTRODUCTION**

After calving, dairy cattle expend tremendous amounts of energy in milk production, which is often not offset by energy intake. When energy expenditure exceeds intake, cows are in negative energy balance (Van Arendonk, 1991). During the transition period, negative energy balance is prevalent and energy substrates in adipose tissue are mobilized to compensate for the energy deficit. Changes in body condition scores and weights have been used as on-the-farm indicators of negative energy balance. However, these indicators represent past *in vivo* events and do not represent current cellular activity. Studies at the cellular level have focused on response to  $\beta$ -agonists and measures of cell size.

Adipose tissue is a highly innervated and active tissue which responds to both autocrine (e.g. leptin and adiponectin) and endocrine hormones (e.g. insulin and catecholamines). Adipose tissue and adipocyte cultures have been treated with catecholamines and other synthetic  $\beta$ -adrenergic receptor agonists to determine  $\beta$ -adrenergic receptor sensitivity. During times of energy deficit, it is expected that  $\beta$ -

adrenergic sensitivity is increased (Sumner and McNamara, 2007) as a mechanism to facilitate mobilization of energy reserves. However, this result has not been consistently observed in studies in dairy cattle. For example, McNamara et al. (1992) observed increased  $\beta$ -adrenergic sensitivity prior to calving, when energy balance is expected to be positive, compared to times of negative energy balance (5-60 DIM).

Adipocyte diameter can be useful as an indicator of energy status because the size of adipocytes decreases upon mobilization of energy reserves. Cows in late pregnancy had similar adipocyte size at -5, -15, -30d relative to calving (McNamara et al., 1992). However, when the same cows were evaluated at 5 DIM, adipocyte diameter significantly decreased, and remained smaller at the last sampling time of 60 DIM.

Characterizing cellular activity during times of varying lipolytic activity will allow for a better understanding of proteins associated with lipolysis. Therefore, our first objective was to determine if  $\beta$ -adrenergic sensitivity differs between early and mid lactation cows. It was expected that adipocytes collected from cows in early (5-14 DIM) lactation have greater sensitivity to isoproterenol compared to adipocytes isolated from cows in mid (176-206 DIM) lactation. Our second objective was to determine if adipocyte diameter differed between cows in early and mid lactation. It was expected that adipocyte diameter would be less in cows in early (5-14 DIM) compared to mid (176-206 DIM) lactation as a result of adipose tissue mobilization.

## **4.2 MATERIALS AND METHODS**

### **4.2.1 Animals**

Twenty Holstein cows of similar body condition scores (2.75-4.5) and lacking disease and severe lameness were selected for this study. Eleven cows were in early lactation (5-14DIM) and nine were in mid lactation (176-206 DIM). Negative energy balance is most severe in early lactation at approximately 5-14 DIM. As cows reach 175 DIM, they are expected to have returned to positive energy balance. Selecting cows prior to 210 DIM allowed for all cows to be fed similar diets at the Iowa State University Ankeny Dairy (Ankeny, IA).

### **4.2.2 Sample Collection**

Approximately 15-25 g of adipose tissue was collected once per cow from the tailhead region, and rinsed in a warmed (37°C) buffered saline solution supplemented with glucose (15mM Sodium chloride, 1mM HEPES, and 11mM D-glucose). Adipose tissue was then transported to the laboratory in the same buffer. A small sample (0.5-2g) of adipose tissue was also collected for slide preparation. This sample was immediately snap-frozen in liquid nitrogen and then stored at -80°C. All surgical procedures were approved by the Iowa State University Institutional Animal Care and Use Committee. Following completion of the biopsy, blood was collected via jugular venipuncture.

### **4.2.3 Culture of Adipose Tissue Explants**

Within 1 hour of removal from the cow, adipose tissue was placed in a warmed buffered saline solution (15mM Sodium chloride and 1mM HEPES). Adipocytes were harvested with protocols adapted from Liu et al. (1989) and Mills et

al. (2003) with a few modifications. Briefly, adipose tissue was chopped and digested in warmed Collagenase cocktail (Krebs-Ringers solution [20mM Sodium bicarbonate, 20mM HEPES, 10mM D-glucose], 1M Sodium bicarbonate, 1 M HEPES, 1 M Glucose, 6% BSA, 2.5mg/ml Collagenase Type I). After digestion, adipocytes were immediately filtered and supplemented with warmed growth media (1000 mg/L DMEM, 4.4 mM Sodium bicarbonate, 5mM HEPES and 3% BSA).

Adipocytes in growth media collected from a single cow were divided among vials supplemented with isoproterenol ranging in concentration from  $1.0 \times 10^{-5}$  M to  $1.0 \times 10^{-10}$  M, or without supplementation. Vials were filled with 20 percent carbon dioxide, 5 percent oxygen and balanced with nitrogen. After 90 minutes of gyrating incubation, media was removed and stored at  $-80^{\circ}\text{C}$  pending analysis for glycerol concentration. Adipocytes were rinsed in PBS and stored in a PBS solution containing 5% of (50% ethanol), vortexed for 10 seconds and stored at  $-20^{\circ}\text{C}$  pending analysis for DNA as an indicator of cell number.

Glycerol concentration in culture media was measured as an indicator of stimulated lipolytic activity. Glycerol was measured using free glycerol reagent (Sigma, F6428) according to manufacturer's protocol. Ninety-six well plates were read at the absorbance of 540 nm by a Tecan Spectrafluor Plus (Tecan Group Ltd.). All samples were run in duplicate. DNA was used to normalize glycerol concentration to a standard number of cells cultured in each vial. DNA was quantified using DNA Quanti-iT dsDNA Assay kit, Broad Range (Molecular Probes, Q33130) according to manufacturer's protocol. All samples were run in duplicate in 96-well plates and read at 485 nm excitation and 530 nm emission on Tecan

Spectrafluor Plus (Tecan Group Ltd.). All data were expressed as glycerol release (mM equivalent of triolien) / DNA (ng/ $\mu$ l).

#### **4.2.4 Haemotoxyin Staining of Adipose tissue**

Frozen adipose tissue samples were mounted in 3-4 $\mu$ m sections on poly-l-lysine coated slides by Iowa State University Pathology and Necropsy Services. Slides were kept frozen until staining. Slides were fixed with 5% formalin for 5 minutes then rinsed three times with PBS and stained with 1 to 1.5 ml of Haemotoxyin for one hour and a half. Slides were then rinsed with PBS and rinsed with running water for 3 minutes. Ninety-five percent glycerol was placed over the sample, and a cover slip was added and fixed for later viewing.

#### **4.2.5 Adipocyte Size**

Adipocyte diameter was measured on five different slides for each cow. Five views from random fields were evaluated for each slide, for a total of 25 views per cow. Adipocytes were viewed on a Zeiss AxioCam light microscope and measured with the accompanying software. Only completely viewed adipocytes in each slide were included, and adipocyte diameter was measured in a north to south direction.

#### **4.2.6 Statistical Analysis**

Lipolytic response of isolated adipocytes stimulated with isoproterenol was evaluated by two approaches. First, glycerol release at a single isoproterenol concentration ( $10 \times 10^{-8}$ M) was evaluated. This dose of isoproterenol was chosen because it falls within the linear portion of the dose response curve for all cows. Glycerol release in the media was evaluated using the PROC MIXED procedure of SAS (SAS, 1999). The mixed model included stage of lactation as a fixed effect and

biopsy week and cow as random effects. Cow was included as a random effect to appropriately account for glycerol measurements from duplicated vials of cells for each cow. The method described by Kenward and Roger was used to determine denominator degrees of freedom (1997).

Second, the lipolytic response of isolated adipocytes stimulated by isoproterenol was analyzed by comparing the dose response curve of adipocytes from cows in early versus mid lactation. A dose response curve was generated for cells representing each cow using GraphPad Prism 5.00 (GraphPad Prism for Windows, 2007) software, fitting and analyzing parameters of the nonlinear regression of XY. The corresponding slope,  $EC_{50}$ , maximal response, and baseline response parameters were calculated. Each parameter representing the dose response curve was evaluated using the PROC MIXED procedure of SAS (SAS, 1999). The mixed model included stage of lactation as a fixed effect and day of biopsy as a random effect. The maximal response value was not estimable for cells from 7 early and 1 mid lactation cow. Therefore, analyses of the dose response curve parameters were repeated with these cows excluded.

Adipocyte diameter was analyzed by two approaches. The first approach compared the means of early and mid lactation cows. Adipocyte size was averaged across views (5) per slide. The 5 slides per cow were analyzed as repeated measures. The means were compared using PROC MIXED of SAS (SAS, 1999). The mixed model included stage of lactation as a fixed effect with biopsy week and cow as random effects as well as slide within cow as a random effect. The method described by Kenward and Rogers was used to determine denominator degrees of

freedom (1997). The second approach divided cells into 10  $\mu\text{m}$  ranges (i.e. 0-10.00  $\mu\text{m}$ , 10.01-20.00  $\mu\text{m}$ , etc.). Adipocytes over 130  $\mu\text{m}$  were grouped together and all adipocytes were expressed on a percentage basis. The number or percent of cells in each size range was evaluated using PROC MIXED of SAS (SAS, 1999). The mixed model included stage of lactation as a fixed effect with biopsy week and cow as random effects. The method described by Kenward and Rogers was used to determine denominator degrees of freedom (1997). Correlations were analyzed using PROC CORR in SAS (SAS, 1999).

### 4.3 RESULTS

Milk production, body condition scores and lipolytic indicators for the cows used in this study have been described previously. The quantity of adipose tissue collected for tissue explants did not significantly differ between early and mid lactation ( $P=0.3384$ ).  $\beta$ -adrenergic sensitivity did not differ between early and mid lactation cows at an isoproterenol concentration of  $1.0 \times 10^{-8}\text{M}$  ( $P<0.05$  ; See Figure 1).

The slope of all cows (See Figure 2 A), maximal response, baseline response, and log of the  $\text{EC}_{50}$  were not significantly different between early and mid lactation cows ( $P>0.05$ ; See Table 1). Correlations of the parameters of the dose-response curve with lipolytic indicators were not significant for either stage of lactation ( $P>0.05$ ; See Table 2 A). Results using only cows with estimates for all dose response curve parameters yielded no significant differences between early and mid lactation cows (See Table 1). However, correlations between parameters of the dose response curve revealed a significant correlation between baseline



response and slope in early lactation (See Table 2b), but parameters of the dose response curve were not significant with lipolytic indicators.

Average adipocyte diameter did not differ between early and mid lactation cows (See Figure 3). In order to better understand the data, a second method was used to determine the distribution of the adipocytes (See Figure 4). Early lactation cows had a significantly greater percentage of adipocytes between 10.01  $\mu\text{m}$  to 20.00  $\mu\text{m}$ . However, as adipocyte percentages did not significantly differ in ranges around 10.01  $\mu\text{m}$  and 20.00  $\mu\text{m}$ , (See Figure 4) this is likely a spurious result. Percentage of adipocytes at the various ranges was not significantly correlated with lipolytic indicators or with parameters representing the dose-response curve (See Table 3).

## 4.4 DISCUSSION

Beta agonists, such as isoproterenol, allow for *in vitro* analysis of  $\beta$ -adrenergic receptor response in cultured cells. Isoproterenol is a non-selective  $\beta$ -agonist that allows  $\beta$ -adrenergic receptor activity to be measured through the release of glycerol and/or NEFA. Previous work from this study showed that  $\beta$ -adrenergic receptor 2 mRNA abundance is significantly increased in adipose tissue of cows in early compared to mid lactation (Chapter 2). Thus, it was hypothesized that a greater response to  $\beta$ -adrenergic receptor activation would be observed in early lactation cows, due in part to an increase in the number of  $\beta_2$  receptors. However, no significant difference was observed.

Others have variable results for the response of adipose tissue explants of isolated adipocytes to  $\beta$ -agonists throughout lactation. McNarama and Hillers

(1986) observed the expected result where early lactation cows had increased response to  $\beta$ -agonists compared to non-lactating cows. However, McNamara et al. (1992) later described an increased response to  $\beta$ -agonists during the dry period compared to lactating cows, but variations were great enough that responses were not significantly different (See Figure 5 for graphical depiction of published data). It should be noted that response to  $\beta$ -receptor activation is highly variable, resulting in large standard errors.

The use of adipose tissue explants compared to isolated adipocytes result in greater variation. Adipose tissue explants result in a spike in response (Sumner and McNamara, 2007; Elkins D. unpublished), whereas adipocytes result in a uniform dose-response curve (Figure 4). Also, isolated adipocytes are often more repeatable within cow compared to adipose tissue explants. However, the method used to isolate adipocytes may result in an improved dose response curve, but may result in diminished *in vivo* conditions from extra processing.

Cows in early lactation that are in negative energy balance are mobilizing adipose tissue resulting in decreases in adipocyte size, whereas cows in positive energy balance are storing triacylglycerides which would result in an increase in adipocyte size. Decreased adipocyte size has been reported at 5 DIM compared to 3 pre-partum similar sampling periods (-30d, -15d, -5d) but adipocyte size was similar for samples taken at 5 and 60 DIM (1992). Average adipocyte size in this study did not significantly differ between early and mid lactation cows. This may be due in part to the selection of cows with similar body condition scores. Additionally, adipocytes in mid lactation cows had likely not reached their maximal lipid

accumulation, as would be expected for adipocyte sampled just prior to calving (McNamara et al., 1992).

## 4.5 CONCLUSIONS

Cellular responses to increased energy mobilization, as indicated by  $\beta$ -adrenergic receptor response and adipocyte size, were similar between early and mid lactation. However,  $\beta$ -adrenergic receptor 2 mRNA abundance and lipolysis were increased in early lactation cows compared to mid lactation. These results may reflect the importance timing on  $\beta$ -adrenergic receptor response and changes in adipocyte size, and that isolated adipocytes may not retain their *in vivo* characteristics.

## 4.6 REFERENCES

- GraphPad. 2007. Graphpad prism for windows. GraphPad Prism, San Diego.
- Kenward, M. G., and J. H. Roger. 1997. Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics* 53: 983-997.
- Liu, C. Y., J. L. Boyer, and S. E. Mills. 1989. Acute effects of beta-adrenergic agonists on porcine adipocyte metabolism in vitro. *J Anim Sci* 67: 2930-2936.
- McNamara, J. P., B. Becker-Khaleel, and K. L. Parmley. 1992. Quantitative relationships between cyclic adenosine-3',5'-monophosphate and lipolysis in adipose tissue during the peripartum period. *J Dairy Sci* 75: 1901-1913.
- McNamara, J. P., and J. K. Hillers. 1986. Regulation of bovine adipose tissue metabolism during lactation. 2. Lipolysis response to milk production and energy intake. *J Dairy Sci* 69: 3042-3050.
- Mills, S. E., M. E. Spurlock, and D. J. Smith. 2003. Beta-adrenergic receptor subtypes that mediate ractopamine stimulation of lipolysis. *J Anim Sci* 81: 662-668.
- SAS (Editor), 1999. Sas/stat user's guide version 6.12. SAS Institute Inc., Cary, NC.

- Sumner, J. M., and J. P. McNamara. 2007. Expression of lipolytic genes in the adipose tissue of pregnant and lactating holstein dairy cattle. *J Dairy Sci* 90: 5237-5246.
- Van Arendonk, J., G. Niewof, H. Vos and S. Korver. 1991. Genetic aspects of feed intake and efficiency in lactation dairy heifers. *Livest. Prod. Sci* 29: 263-275.

Figure 1: *Early (5-14 DIM) and Mid (176-206 DIM) lactation adipocyte response to log -8M isoproterenol concentration.* This concentration of isoproterenol was selected as the concentration that fell on the linear curve. Early lactation is represented by (■). Mid lactation is represented by (□).

Figure 2: *Dose-response curve of cows isoproterenol stimulation.* **A.** Early (5-14 DIM) (dashed line) and Mid (176-206 DIM) lactation (solid line) of all cows at all isoproterenol concentrations. **B.** Early and Mid lactation cows with all values along the slope. The isoproterenol concentration of  $1.0 \times 10^{-12} \text{M}$  represents vials lacking isoproterenol supplement. This image was generated from GraphPad Prism 5.00 and this program does not allow for input in non-numerical form.

Figure 3: *Average adipocyte size by stage of lactation.* Early (5-14 DIM) lactation is depicted as (■) and mid (176-206 DIM) lactation depicted as (□). Adipocytes were averaged from 5 fields to create 5 averages per cow.

Figure 4: *Distribution of the percentage of adipocyte in Early (5-14 DIM) and Mid (176-206 DIM) lactation cows.* Adipocytes were measured in the north to south direction and only adipocytes that were completely in the microscope view were included (inset). Adipocytes were summed from 5 slides with 5 different views per cow and expressed as a percentage of adipocytes within a  $10 \mu\text{m}$  range. Early lactation is represented by (■). Mid lactation is represented by (□).

\*  $P < 0.05$

Figure 5: *Graphical depiction of data from McNamara et al. (1992) and McNamara and Hillers (1986).* **A.** Data from McNamara et al. (1992). (▲) indicates a 15 minute incubation. (■) indicates 2 hour incubation. **B.** Data from McNamara and Hillers (1986). (▲) represents high genetics, (x) represents low genetics, (◆) represents high diet, and (■) represents low diet. For further explanation of the data presented refer the indicated studies.

Figure 1

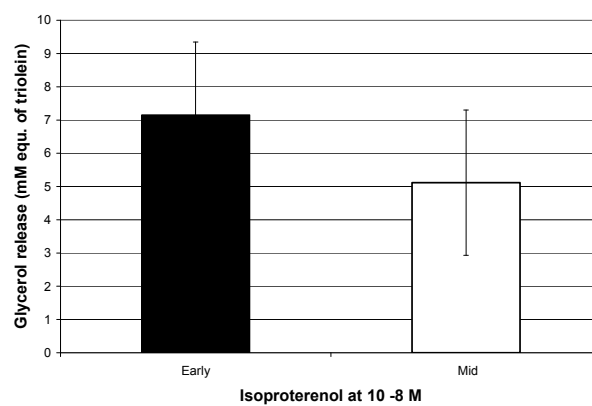
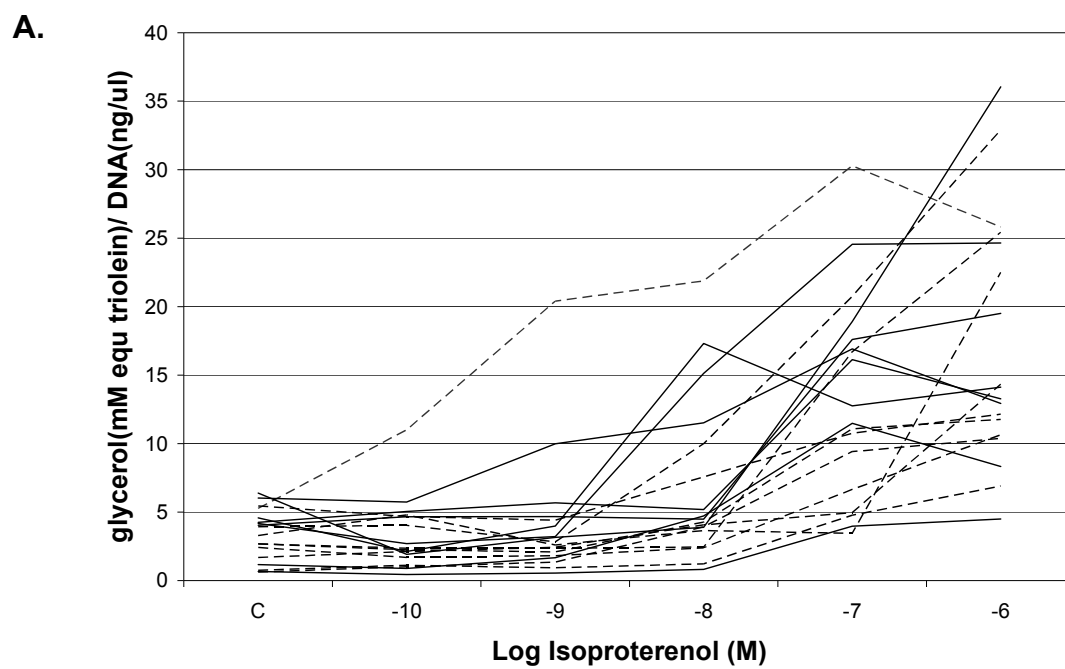


Figure 2



**B.**

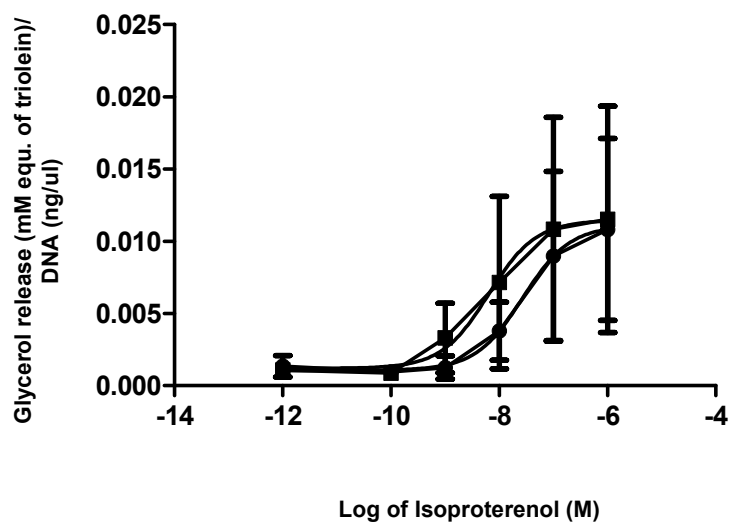




Figure 3

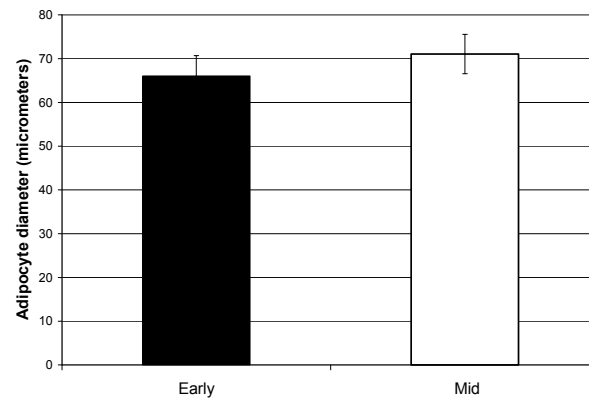


Figure 4

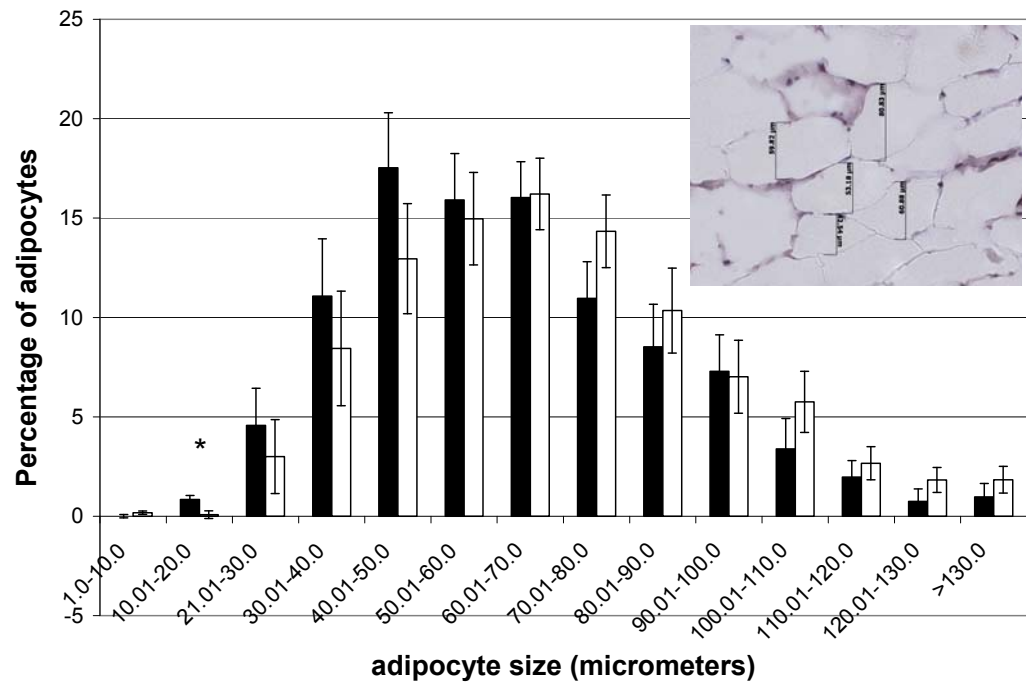


Figure 5

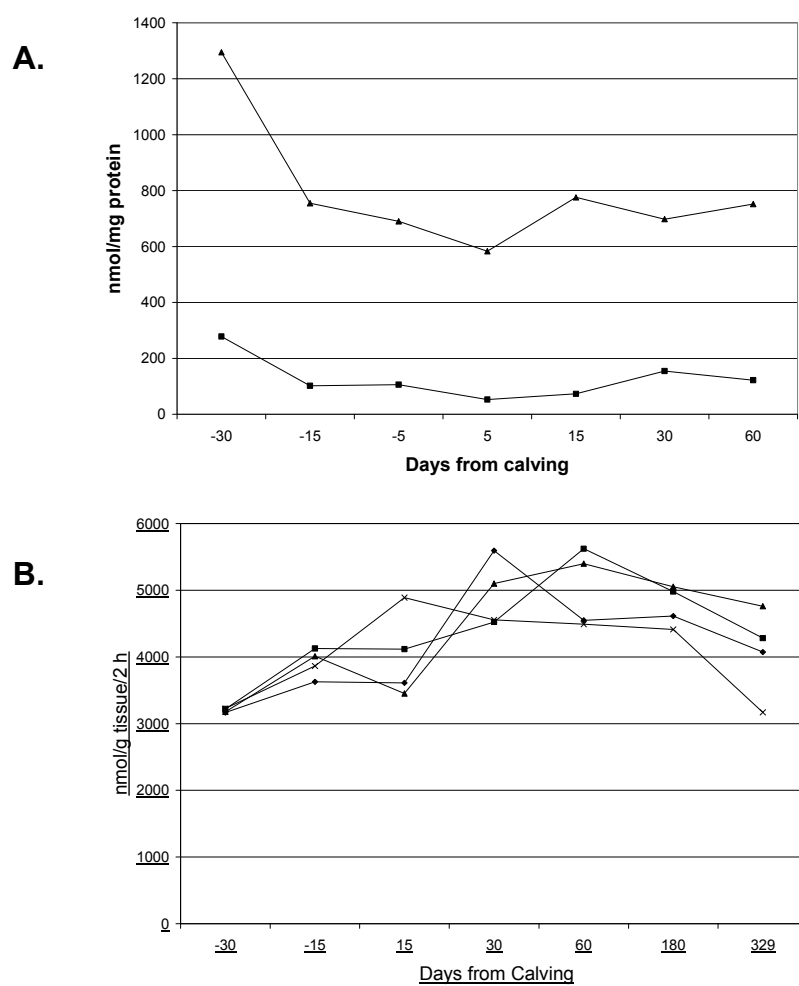


Table 1: Parameters of the dose-response curve of Early (5-14DIM) and Mid (176-206DIM) lactation adipocytes when stimulated with isoproterenol.

	N <sup>a</sup>	Early	SEM	N	Mid	SEM	P-value
Slope <sup>b</sup>	11	2.44	1.17	7	5.39	1.32	0.07
Maximal	4	4.12	1.03	6	3.38	0.86	0.56
Baseline	11	3803.7	2926.5	7	15.12	3497.8	0.42
Log EC <sub>50</sub>	11	-7.44	0.32	7	-7.84	0.36	0.40
Slope	4 <sup>c</sup>	3.92	2.13	6	6.30	1.84	0.32
Maximal	4	4.12	1.03	6	3.38	0.86	0.56
Baseline	4	19.23	3.61	6	13.49	3.01	0.22
Log EC <sub>50</sub>	4	-8.35	0.40	6	-7.96	0.33	0.49

<sup>a</sup> abbreviations: N, number of cows; Early, early lactation cows; SEM, standard error of the mean; Mid, mid lactation cows;

<sup>b</sup> Slope is the measure of glycerol release by adipocytes in response to the concentration of isoproterenol administered to adipocytes cultures generated by GraphPad Prism 5.0 software®; Maximal response is the measure of the upper most value on the dose-response curve generated by GraphPad Prism 5.0 software®; Baseline response is the amount of glycerol release when not stimulated by isoproterenol as calculated by GraphPad Prism 5.0 software®; Log EC<sub>50</sub> is the log of fifty percent of the effective concentration generated by GraphPad Prism 5.0 software®

<sup>c</sup> analysis of cows that had all values (slope, maximal, baseline, Log EC<sub>50</sub>)

Table 2: Correlations of parameters of the isoproterenol dose-response curve of Early (5-14 DIM) and Mid (176-206 DIM) lactation cows. Early lactation correlations are below the diagonal. Mid lactation correlations are above the diagonal.

A.	All Cows			
	Slope	Log EC <sub>50</sub>	Max.	Base.
Slope <sup>a</sup>		0.57	0.18	0.02
Log EC <sub>50</sub>	0.61		0.04	0.09
Max.	0.33	-0.75		0.70
Base.	0.32	0.28	0.52	

B.	Cows <sup>b</sup>			
	Full Slope	Slope Log EC <sub>50</sub>	Max.	Base.
Slope <sup>a</sup>		-0.48	-0.18	0.27
Log EC <sub>50</sub>	-0.64		0.04	-0.31
Max.	0.33	-0.75		0.70
Base.	0.97*	-0.78	0.52	

<sup>a</sup> Slope is the measure of glycerol release by adipocytes in response to the concentration of isoproterenol administered to adipocytes cultures generated by GraphPad Prism 5.0 software®; Max., Maximal response is the measure of the upper most value on the dose-response curve generated by GraphPad Prism 5.0 software; Base., Baseline response is the amount of glycerol release when not stimulated by isoproterenol as calculated by GraphPad Prism 5.0 software®; Log EC<sub>50</sub> is the log of fifty percent of the effective concentration generated by GraphPad Prism 5.0 software®

<sup>b</sup> analysis of cows that had all values (slope, maximal, baseline, Log EC<sub>50</sub>)

## **CHAPTER 5: GENERAL DISCUSSION**

### **5.1 REVIEW OF DAIRY INDUSTRY TRENDS**

The dairy industry widely recognizes trends of increased milk production per cow per lactation in recent years. Often increases in milk production increases the prevalence of negative energy balance during early lactation. Ketosis and milk fever have been associated with negative energy balance. Increased milk production and negative energy balance has been associated with decreased reproduction (Dechow et al., 2004), increased laminitis (Collard et al., 2000), and decreased udder health (Banos et al., 2006). Therefore, better understandings of energy mobilization during early lactation will be helpful to develop strategies for preventing decreased fitness traits associated with negative energy balance.

The PKA pathway has many important roles in energy mobilization in adipose tissue. Immunofluorescence has been used to describe movements of the PKA pathway, shaping the current pathway (Figure 1). However, several components of the PKA pathway include perilipin, ATGL, and CGI-58 have been almost exclusively characterized in various cell lines, in rodents, and in human case studies. To date, these proteins have received little attention in dairy cattle, but could be key in developing pharmaceuticals or breeding strategies to prevent extreme or prolonged negative energy balance.

### **5.2 SUMMERIZATION OF RESULTS AND DISCUSSION**

The current research in dairy cattle has identified possible mechanisms critical for lipid mobilization in dairy cattle, and has added knowledge of newly

associated proteins in a divergent species. With the use of monoclonal antibodies specific to PKA phosphorylated serine sites of HSL, the PKA pathway was confirmed to be significantly increased during early lactation and correlated lipolytic indicators. Significant correlations of HSL protein, but not mRNA with lipolytic indicators in early lactation cows might suggest that abundance and translation of HSL might be rate limiting steps in bovine PKA dependent lipolysis.

Since average adipocyte diameter size does not differ between early and mid lactation cows in this study allows for a clearer understanding of the role of perilipin abundance in lipolysis of dairy cattle. Research has suggested that similar abundance of perilipin at the lipid droplet of differing adipocyte diameters may serve a role in lipolysis. However, in dairy cattle of similar adipocyte size as presented in this study, perilipin abundance does not change with changes in lipolytic activity. However, phosphorylation of perilipin by PKA is significantly increased during early lactation and correlated with lipolytic indicators during both early and mid lactation. Phosphorylation of perilipin by PKA is a possible rate-limiting step of perilipin involvement in dairy cattle.

Adipose triglyceride lipase is a newly associated lipase with stimulated lipolysis in rodent models and cell culture, but may not be with all species. At this time, direct measurements of ATGL activity are not quantifiable except with HSL knock-out or knock-down models. However, unlike other PKA associated components in this study, ATGL abundance was increased in mid lactation cows which could indicate increased ATGL activity. Interestingly, ATGL abundance was not correlated to lipolytic indicators in either early or mid lactation cows. This may

indicate that different ATGL regulation is PKA independent, or that ATGL plays a different role in PKA pathway in dairy cattle. The increased ATGL abundance during low lipolytic activity could be needed for increased availability for decreased CGI-58 translocation. However, until ATGL protein abundance is correlated with ATGL activity, many theories can be suggested but not proven.

Results presented in this study have shed light on lipolytic activity through the PKA pathway from different states of lactation in dairy cattle. Additionally, this study has associated many gene transcriptions of the PKA pathway. Perilipin, HSL, and  $\beta$ -AR mRNA abundances are correlated during early and mid lactation. Both perilipin and HSL have been identified to possibly contain PPAR response elements (PPRE) suggesting coordinated transcription (Yajima et al., 2007). Although, mRNA abundance not correlate with corresponding protein abundance suggesting microRNAs may prevent translation. At this time, no literature links  $\beta$ -AR with PPRE, but correlations with perilipin and HSL mRNA suggest a similar time of transcription. Messenger RNA of ATGL is significantly correlated with perilipin mRNA and HSL mRNA abundance during early lactation and with tended to be correlated with HSL mRNA abundance during mid lactation (See Appendix). Literature has not determined if a PPRE site exists with ATGL, and with the inconsistencies in correlation, it is not likely. However, ATGL is regulated by PPAR $\gamma$ , and may cause the correlation of ATGL with perilipin and HSL mRNA during early lactation.



### 5.3 LIMITATIONS

This research has provided useful insight in the role of the PKA pathway during early and mid lactation; although, there were several limitations that limit the use of the data. A limitation was the lack of feed intake data. Had feed intake been available, energy balance could have been calculated for cows used in this study. Energy balance data would allow for a better understanding of the various rates of lipolysis with various energy balances. The current study used animals of early and mid lactation, but without energy balance data, only lipolytic indicators can be used to predict lipolytic activity.

Another limitation of this study was the small sample size and block sampling (one early and one mid lactation cow per biopsy day). Selection criteria for cows used in this study inadvertently limited the number of cows. The selection basis of 5-15 DIM (early lactation) was selected to capture cows in negative energy balance, however; we were restricted to the number of cows bred which occurred prior to this study. The selection basis of 175-200 DIM (mid lactation) was initially selected, however; due to the summer heat, cows were not accumulating the amount of tissue needed for the biopsies. As cows used in this study were housed in a farm setting, and are prone to disease, this resulted in the exclusion of cows with cases of mastitis or severe lameness during the current lactation. We selected to sample cows in early and mid lactation pairs to reduce the day of sampling effect. Although, for explant culturing of adipocytes, a few cows biopsied failed to produce viable adipocytes. For this reason, cows were selected for western blotting and serum measurements on the basis of the viability of adipocyte harvested.

A final limitation of this study was the use of different cows for the different stages of lactation. Therefore, this introduced greater variability of expected energy mobilization. This is demonstrated with the significant increase in 305d mature equivalent milk production, milk protein, and milk fat of the early compared to mid lactation cow. This variation prevents fine detailing and possible correlations that are essential for developing methods of prevention of severe and prolonged negative energy balance. However, this limitation also has a benefit. Due to the variation amongst cows, it is easier to understand overall mechanism of lipolysis in dairy cattle.

## 5.4 FUTURE RESEARCH

For more insight into understanding individual differences in lipolysis an in-depth study is needed to address the effects of these novel protein on energy balance. *An overall goal of future research would be to determine the amount of variation of phosphorylation of perilipin and HSL, and to determine protein abundance of ATGL and CGI-58 during different energy balance across multiple milk production ranges.* Measurements of feed intake, milk production, and milk components would be needed to calculate estimated energy balance of cows until positive energy balance is expected. To characterize *in vivo* energy indicators (NEFA, glycerol, and  $\beta$ -Hydroxybutyrate), serial serum collection would be needed. Glycerol, NEFA, and  $\beta$ -Hydroxybutyrate do not change postprandially and are good overall energy indicators. External measures of energy status can be observed by weekly body weight and weekly body condition scores. With lipolytic indicator, energy balance, body weigh, and body condition scores, a timeline can be

established on a week basis of energy usage. It would be expected that NEFA and glycerol concentration would increase just prior to or around calving (Accorsi et al., 2005) indicating increased energy needs to produce milk.  $\beta$ -Hydroxybutyrate is an indicator of ketosis; therefore, the concentration of  $\beta$ -Hydroxybutyrate would only increase in cows with prolonged or severe negative energy balance. It would be expected cows in a sub-clinical or acute ketosis would be observed more frequently than cows with clinical ketosis.

#### **5.4.1 Adipose Tissue Sampling Times**

*A specific goal of this future project would be to collect multiple samples of adipose tissue during peak negative energy balance and at the beginning of recovery from negative energy balance.* Sampling times and number of samples during lactation would need to be carefully chosen especially when using repeated sampling from cows. To fully understand the effects of negative energy balance on energy mobilization, it would be ideal to collect an adipose tissue sample during individual low points of energy balance. However, individual low points are not known to the researchers until much later. Based on literature, cows are at their lowest energy intake and highest energy output during the first 14 DIM (Patton et al., 2007), and are beginning to reach neutral energy balance as early as 30 DIM (Accorsi et al., 2005). Therefore, an ideal range of DIM to capture of highest *in vivo* lipolytic activity would be during or prior to nadir approximately 5-10 DIM. To determine the point cows begin to down-regulate lipolytic activity requires a similar retrospective viewing of feed intake, milk production, and lipolytic indicators. With energy balance on the rise following nadir, adipose tissue should exhibit down-

regulation of mechanism involved in mobilization of adipose tissue by 21-30DIM. An adipose tissue sample would need to be collected while the cow is in positive energy balance. This could be while the cow is dry, or as the cow approaches 150-200DIM. Consideration of season and availability of adipose tissue make it more likely that sampling during the dry period would be most optimal. Prior to calving or at calving, cows begin will show spikes of NEFA. For this reason and the variation of predicted calving dates and calving, adipose tissues would be optimally sampled at 30 to 21 d prior to calving.

#### **5.4.2 PKA Pathway**

To further understand lipolysis in dairy cattle and its role in adipose tissue mobilization during early lactation quantification of mRNA and protein of components along the pathway should be investigated. Many proteins associated with the PKA pathway are not consistently correlated with their mRNA transcripts. *A second specific goal would be to quantify both RNA and protein abundances of perilipin, HSL, ATGL and CGI-58 to determine if environmental cues are upregulating gene transcription or if internal inhibitors of translation are decreased during early lactation.* Quantification of perilipin, HSL, ATGL, CGI-58, and  $\beta$ -AR<sub>2</sub> mRNA abundance from the dry period throughout early lactation will provide insight to possible shifts in protein requirements. However, since mRNA and protein are not well correlated, it is not likely to determine protein abundance. Although, this data would provide information concerning conserved transcription of genes. This would allow for a better understanding of gene regulation during early lactation of genes/proteins involved in lipolysis. Quantification of mRNA abundance using

quantitative real-time PCR could determine possible variation between transcripts in cows that undergo extreme or little negative energy balance. These variations could be due to causative mutations that prevent protein translation or from hormonal variations. Of the proposed genes, I would hypothesize that HSL and  $\beta$ -AR<sub>2</sub> mRNA abundance would increase with lipolysis, that ATGL mRNA abundance would decrease through early lactation and that perilipin and CGI-58 mRNA abundance would remain constant throughout early lactation.

From this study, the importance of protein abundance and especially phosphorylation abundance was determined for perilipin and HSL. Protein abundance of HSL and phosphorylation of perilipin were associated with lipolytic indicators. To determine if energy balance is associated with changes in protein and phosphorylation, protein abundance of HSL, ATGL, CGI-58, perilipin and phosphorylated HSL and perilipin should be measured via semi-quantitative western blotting. Quantification of protein and phosphorylation abundance could potentially develop a timeline of the PKA-dependent lipolysis during the transition period. Dry cows would be in neutral or positive energy balance and would not be mobilizing adipose tissue. During this time, protein abundance would be expected to be similar to early lactation but phosphorylation would be significantly decreased. At 5-10 DIM, adipose tissue would begin to be mobilized and phosphorylation by PKA would be increased. As cows pass nadir, adipose tissue becomes less of an energy source, so phosphorylation by PKA would be decreased compared to the 5-10DIM, but still greater than the prior to calving.

### 5.4.3 Hormonal Influences on the PKA Pathway

It is expected that hormones provide cues to adipocytes that result in changes of internal conditions. *A third specific goal would be to characterized hormones that are associated with energy partitioning, and determine the possible effects these hormones have on lipolysis.* Hormonal regulation of lipolysis during the transition period can be measure by serum assays. The hormones responsible for initiating the PKA pathway, epinephrine and norepinephrine, would provide insight into the amount of signaling to adipose tissue. It would be expected as epinephrine concentration in serum increases with negative energy balance. Variation of epinephrine and norepinephrine concentrations could greatly vary among cows as they continue into mid and late lactation (McNamara and Hillers, 1986) allowing for a better understanding of regulatory differences in cow with prolonged negative energy balance. A possible question to be answered would be: if cows in prolonged energy balance have increased catecholamine response, does the PKA pathway remain active allowing for more depletion of adipose tissue, or do perilipin, HSL, ATGL or CGI-58 down regulate lipolysis?

Leptin and adiponectin are adipocyte secreted hormones (adipokines) that are associated with postprandial energy status. Both adipokines act as autocrines and paracrines. As autocrines, leptin and adiponectin bind receptors that activate the AMPK pathway. In adipose tissue, the AMPK pathway activates triacylglyceride synthesis and inhibits lipolysis. Leptin concentrations decrease during early lactation (Accorsi et al., 2005), but adiponectin concentrations greatly vary in amount and pattern of change (Spurlock,D., unpublished). The associations of adipokines

that can inhibit lipolysis and sense energy balance could possibly identify a feedback system in adipose tissue when lipolysis becomes extreme. Leptin and adiponectin, also, act as paracrine hormones secreted from adipose tissue. Leptin is able to cross the blood brain barrier and signal of satiety. Decreases following calving would indicate to the brain an insufficient supply of feed intake. With adiponectin serum levels varying during transition, it may play a key role in inhibiting lipolysis.

Growth hormone has been shown to increase around calving (Accorsi et al., 2005) and is used pharmaceutically to maintain high milk production following peak lactation. The effects of growth hormone on adipocytes are still under research. It might be expected that increases in growth hormone with early lactation and increases epinephrine are activating multiple lipolytic responses increasing the rate of lipolysis and may desensitize adipocytes quickly which prevents extreme or prolonged negative energy balance in some cows. Semi-quantitative western blotting would be used to measure serum levels of growth hormone throughout early lactation.

*A final specific goal would be to determine the relationship of ATGL abundance with its activity in dairy cattle and the movement of CGI-58 in primary adipocytes.* Direct measurements of *in vivo* ATGL and CGI-58 activity can not be directly measured at this time, a separate *in vitro* study would be needed to understand their possible role in lipolysis and stimulation by various hormones. The possible roles of ATGL and CGI-58 could be investigated two ways. Isolated bovine stromal vascular cells could be cultured and differentiated into adipocytes. Small interfering

RNA for HSL could be used to allow only ATGL lipolytic activity to be observed. Cultures exposed to hormones previously mentioned that may play roles in lipolysis of early lactation could allow for a better understanding of ATGL involvement in lipolysis. With protein and mRNA abundance and diacylglyceride concentration to various stimuli, the relationship of ATGL abundance to ATGL activity could provide a standard method to measure *in vivo* ATGL activity.

Secondly, isolated bovine stromal vascular cells cultured and differentiated into adipocytes on glass covers could be used to determine protein movement of CGI-58 using immunofluorescence under different stimuli. Adipose triglyceride lipase has not been demonstrated to make major movements to and from the lipid droplet. Although, HSL could be immunofluoresced in at similar times as CGI-58 to determine if the movements of CGI-58 and HSL are reversed of each other in cattle as described in rodents (Yamaguchi et al., 2007).

Data gathered from this future project could result in farther extensions of research. As the understanding of lipolysis in dairy cattle increases, it would be projected various reactions to similar concentrations of hormones are responsible for variation in milk production or energy balance between cows. For variations at the pathway level, in particular the PKA pathway, targeted sites of variation could be limited to phosphorylation regions of the perilipin and HSL. Sites of mutations that affect the function of protein have been demonstrated in human for ATGL and CGI-58 which are located in the C-terminus region where emphasis can be directed. Determining proteins or gene regions responsible for severe and prolonged negative

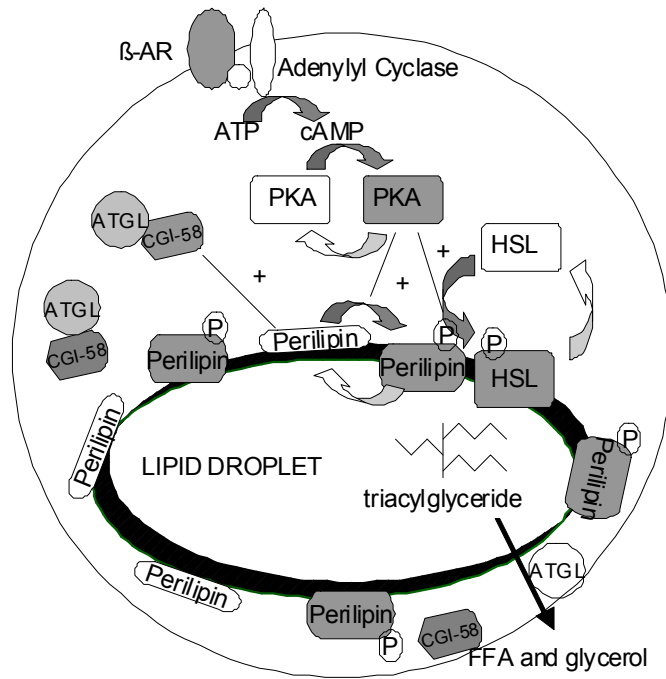


energy balance will enable better breeding strategies and pharmaceuticals to counteract the detrimental effects to fitness traits.

## 5.5 REFERENCES

- Accorsi, P. A. et al. 2005. Leptin, gh, prl, insulin and metabolic parameters throughout the dry period and lactation in dairy cows. *Reprod Domest Anim* 40: 217-223.
- Banos, G., M. P. Coffey, E. Wall, and S. Brotherstone. 2006. Genetic relationship between first-lactation body energy and later-life udder health in dairy cattle. *J Dairy Sci* 89: 2222-2232.
- Collard, B. L., P. J. Boettcher, J. C. Dekkers, D. Petitclerc, and L. R. Schaeffer. 2000. Relationships between energy balance and health traits of dairy cattle in early lactation. *J Dairy Sci* 83: 2683-2690.
- Dechow, C. D., G. W. Rogers, L. Klei, T. J. Lawlor, and P. M. VanRaden. 2004. Body condition scores and dairy form evaluations as indicators of days open in us holsteins. *J Dairy Sci* 87: 3534-3541.
- McNamara, J. P., and J. K. Hillers. 1986. Regulation of bovine adipose tissue metabolism during lactation. 2. Lipolysis response to milk production and energy intake. *J Dairy Sci* 69: 3042-3050.
- Patton, J. et al. 2007. Relationships among milk production, energy balance, plasma analytes, and reproduction in holstein-friesian cows. *J Dairy Sci* 90: 649-658.
- Yajima, H., Y. Kobayashi, T. Kanaya, and Y. Horino. 2007. Identification of peroxisome-proliferator responsive element in the mouse hsl gene. *Biochem Biophys Res Commun* 352: 526-531.
- Yamaguchi, T. et al. 2007. Cgi-58 facilitates lipolysis on lipid droplets but is not involved in the vesiculation of lipid droplets caused by hormonal stimulation. *J Lipid Res* 48: 1078-1089.

Figure 1. *Protein kinase A pathway as characterized in rodents*. Dark gray circle indicate active components of the pathway and plus signs indicate movement. Clear arrows indicate less active movements. (P) indicate phosphorylation of a protein. The movement of CGI-58 into the cytosol is predicted to occur as a result of phosphorylation of perilipin.



## **APPENDIX: ADIPOSE TRIGLCYERIDE LIPASE MESSENGER RNA ABUNDANCE BETWEEN EARLY AND MID LACTATION COWS**

An extension of Chapter 3:

### **A.1 MATERIALS AND METHODS**

#### **A.1.1 Quantitative Real-time PCR**

Total RNA was extracted using the RNeasy Lipid Tissue Midi Kit (Qiagen, 75842) with DNase digestion on the column (RNase-Free DNase set; Qiagen, 79254) and concentrated using the RNeasy MinElute Cleanup kit (Qiagen, 74204), according to manufacturer's protocols. Quantified was measuring by absorbance at 260nm on a Nanodrop (Thermo Scientific). Complimentary DNA (cDNA) was synthesized from 2.5 µg using the MMLV reverse transcriptase protocol (Invitrogen, 28025-013) according to manufacturer's protocols. Quantitative real-time PCR was used to measure mRNA abundance of ATGL and ribosomal protein 32L (housekeeping gene). Primers used for ATGL: forward- 5'- AGC TCA AGA ACA CCA TCA CG -3', reverse- 5'-GTT TGC ACA TCT CTC GAA GC-3'; ribosomal protein 32L: forward- 5' –TCT GGC CCT TGA ATC TTC TG-3', reverse-5'-CCT CGT GAA GCC TAA CAT GC-3'. Samples were run in triplicate on the MyiQ single-color real-time PCR detection system (Bio-Rad) using My iQ SYBR Green Supermix (Bio-Rad, 170-8882) conducted at half volume of manufacturer's protocol.

For absolute quantification, a standard curve was generated using plasmid DNA diluted to produce a range from  $2 \times 10^2$  to  $2 \times 10^8$  copies per  $\mu\text{l}$ . Cycle threshold determined by MyiQ single-color real-time PCR detection system (Bio-Rad) was regressed on the known log of the starting copy number (LSCN) of each transcript's standards. This resulted in regression line that was used to predict the LSCN of all experimental samples which were used for statistical analysis.

## **A.2 RESULTS**

Using q-PCR, mRNA abundance of ATGL did not differ between early and mid lactation cows ( $P > 0.05$ ; Figure 1). Interestingly, mRNA abundance of ATGL was significantly correlated ( $P < 0.05$ ) with mRNA abundance of  $\beta$ -adrenergic receptor 1 and 2, HSL, and perilipin during early lactation and only the correlations of  $\beta$ -adrenergic receptor 1 and HSL tend to be significant in mid lactation ( $P < 0.09$ ; See Table 1).

## **A.3 DISCUSSION**

Interestingly, our study has shown a significant correlation of mRNA abundance of HSL and perilipin with ATGL. In rodent studies, mRNA and protein expression/abundance of HSL and ATGL generate similar patterns from similar treatment (Joken et al., 2007). At the time of this study, no studies address the relationship of perilipin and ATGL mRNA expression/abundance. However, perilipin and ATGL mRNA is expressed upon differentiation, and both are responsive to peroxisome proliferator activated receptor gamma. These results may indicate a common transcription factor responsible for the mRNA production, but that unknown factors are responsible for the aiding or impeding translation.

Figure 1: *mRNA abundance of adipose triglyceride lipase*. Fold change difference between Early (5-14 DIM) and Mid (176-206 DIM) lactation. *Solid bars represent early lactation and open bars represent mid lactation.*

\*  $P < 0.05$

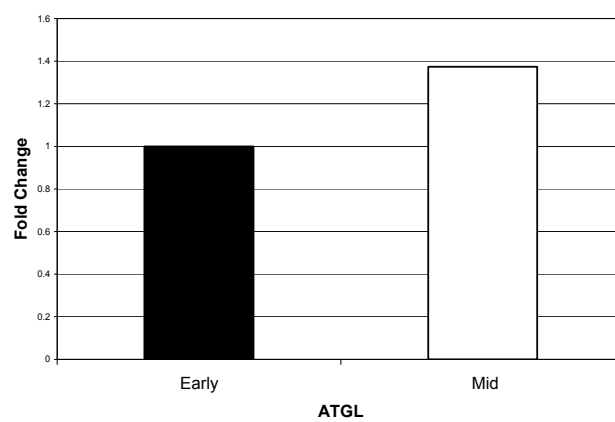


Table 1: Correlations of adipose triglyceride lipase mRNA abundance with perilipin and hormone sensitive lipase at the mRNA, protein and phosphorylated levels and with lipolytic indicators during early (5-14 DIM) and mid (176-206 DIM) lactation

	Early Lactation N <sup>a</sup> =11	Mid Lactation N=9
HSL mRNA	0.82*	0.73
HSL protein	0.15	0.19
PHSL protein	0.35	-- <sup>b</sup>
Plin mRNA	0.93**	0.60
Plin protein	-0.20	0.05
PPlin protein	0.21	-0.07
Glycerol	0.43	0.34
NEFA	0.29	-0.57

\*P<0.05

\*\*P<0.001

<sup>a</sup> Abbreviations N, number; HSL, hormone sensitive lipase; PHSL, phosphorylated hormone sensitive lipase; Plin, perilipin; PPlin, phosphorylated perilipin; NEFA, non-esterified fatty acid

<sup>b</sup> Phosphorylated HSL was not detected during mid lactation and were excluded



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